SYNTHETIC AND BIOSYNTHETIC STUDIES ON FUNGAL METABOLITES

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ABSTRACT

The results of ^{13}C and ^{2}H n.m.r. analyses of [$^{2}H_3$]- and [1,2-$^{13}C_2$]acetate-enriched tajixanthone a xanthone metabolite of *Aspergillus variecolor* indicate that ring cleavage of an anthraquinone, and not anthrone, precursor must precede C-prenylation, and that dihydropyran ring formation precedes xanthone ring formation during biosynthesis of tajixanthone. The stereochemistry of dihydropyran ring formation was studied *in vitro* by synthesising model O-prenyloxy salicylaldehydes and studying their acid catalysed cyclisation. Incorporation of labelled chrysophanol and islandicin into tajixanthone was attempted.

A convenient synthesis of substituted 6-alkoxy-pyran-2-ones was developed which led to an efficient synthesis of ^{2}H and ^{14}C labelled anthraquinones. The Diels-Alder chemistry of these pyrones was further studied. They react readily with acetylenic dienophiles to give a facile route to polysubstituted benzenoid compounds. Problems reported with a previously published synthesis of mevalonic and lactone have been examined and overcome.

The regio- and stereospecificity of incorporation of label from [$^{2}H_3$]acetate into scytalone a dihydronaphthalene metabolite of *Phialaphora lagerbergii* has been determined by high field ^{1}H and ^{2}H n.m.r. studies. Incorporation studies with [2-^{13}C]malonate failed to reveal an acetate "starter" effect suggesting that scytalone may be derived from a hexaketide precursor rather than a pentaketide as previously proposed. This has been further examined by synthesising ^{14}C labelled 1,3,6,8-tetrahydroxynaphthalene and 2-acetyl-1,3,6,8-tetrahydroxynaphthalene and studying their incorporation into scytalone.

ACKNOWLEDGEMENTS

I would like to thank Dr. Tom Simpson for suggesting the areas of research and for his supervision during my studies. Thanks also go to my colleagues in the research school for many helpful discussions and advice on various practical techniques.

The technical staff of the Chemistry Department are to be thanked for the efficient provision of various services. I am especially grateful to Mrs. Suzan Kasperek for carrying out Microbiological work associated with this research. The excellent typing is the work of Mrs. Dorothy Pilling to whom I am very grateful.

CONTENTS

PUBLICATIONS

1. Biosynthesis of Tajixanthone in Aspergillus variecolor: Incorporations of [$^{2}H_{3}$]acetate and [1,2-$^{13}C_{2}$]acetate.

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2. ^{13}C and ^{2}H Labelling Studies on the Biosynthesis of Scytalone in Phialaphora lagerbergii.

   E. Bardshiri and T.J. Simpson, Tetrahedron, 1983, 3539.

3. Studies on a Synthesis of (RS)- Mevalonic Acid Lactone.

   E. Bardshiri, T.J. Simpson, A.I. Scott and K. Shishido, J. Chem. Soc. Perkin Trans. 1, 1984, 1975.

4. Biosynthesis of Tajixanthone and Shamixanthone by Aspergillus variecolor: Incorporation of Oxygen-18 Gas.

   E. Bardshiri, C.R. McIntyre, T.J. Simpson, R.N. Moore, L.A. Trimble and J.C. Vederas, J. Chem. Soc. Chem. Commun., 1984, 1404.

CHAPTER 1

BIOSYNTHESIS OF POLYKETIDES

BIOSYNTHESIS OF POLYKETIDES

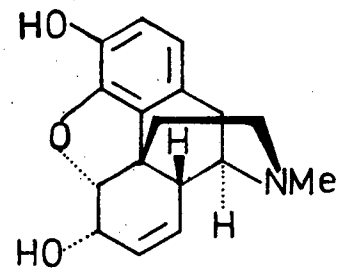
1.1 INTRODUCTION

Normally by biosynthesis we mean the precise ordered sequence of chemical reactions by which a living organism constructs a complex molecule from comparatively simple precursors; however, it can also refer to the formation of a smaller molecule from a larger one. Of course, as we shall see, the initial precursor for all organic material is carbon dioxide.

A natural product is any compound derived from living material and can be anything from the simplest of organic compounds, e.g. CH_4, CH_3OH, HCOOH, CO_2, ethylene or ethanol through more complex substances such as cholesterol, vitamin B_{12}, to the natural polymers, proteins and nucleic acids with thousands or indeed millions of atoms. Each and every one of these compounds is formed in nature by a precise ordered sequence of enzyme mediated chemical reactions known as the biosynthetic pathway.

We can divide natural products into two broad categories, all organisms possess similar metabolic pathway by which they synthesize and utilise certain essential chemicals:- Sugars, amino acids, common fatty acids, heterocyclic bases; and the polymers derived from them:- polysaccharides, proteins, lipids and nucleic acids. This is primary metabolism, and these ubquitous compounds which are essential for survival and well being of all forms of life are called primary metabolites.[2]

However, many organisms also utilise other metabolic pathways producing compounds which usually have no apparent utility to the organism. They are of limited taxonomic distribution, (any specific compound being produced by one species only or a limited number of closely related species). These are called secondary metabolites,[1]



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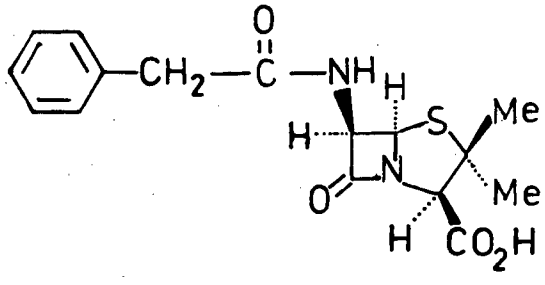

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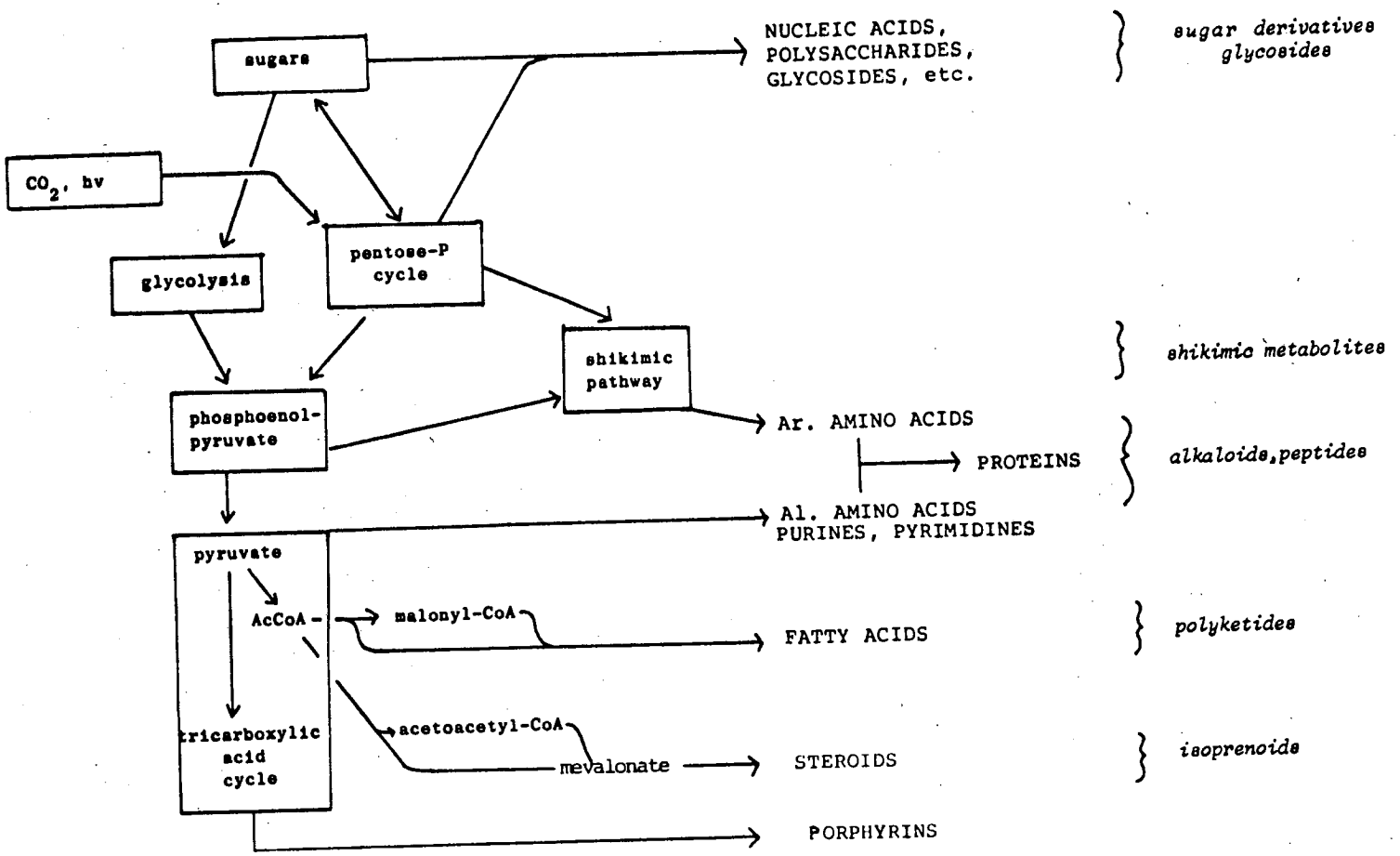


Figure 1 The main flow of carbon metabolism (left), the main products of primary synthesis (capitals), and the corresponding categories of secondary metabolite (italics).

and their pathway of synthesis (and utilisation) constitute secondary metabolism.

Some examples of secondary metabolites will help to clarify the definition. Morphine (1) occurs in only two species of poppy, Papaver Sominiferum and P. Setigerum; and although widely used by man, has no known function in these plants. Similarly, penicillins, e.g. (2) are produced by a few species of fungi but no other organisms; they are invaluable as antibiotics but have no apparent use to the micro-organisms that produce them. On the other hand, man has long found these secondary metabolites of use as medicines, perfumes, spices, poisons, colouring matters and this of course led to them being among the first compounds to be investigated by chemists.

Of course the boundary between primary and secondary metabolism is rather blurred and there are many compounds which are not readily assigned to one category. In addition the two types of metabolism are inter-related, since primary metabolism provides the small molecules which are the precursors for the pathways of secondary metabolism as indicated in Figure 1 which shows the main pathways for the metabolism of carbon.

The small organic molecules which are the building blocks of the biosynthetic pathways and the energy required to maintain metabolism and thus, life itself, comes (in all organisms) from the breakdown of glucose. In all green plants, algae and some bacteria glucose can be produced from CO_2 by photosynthesis via a sequence of reactions collectively known as the pentose-phosphate cycle. In all other organisms, e.g. animals, this glucose is derived from the breakdown of carbohydrates produced by plants and other photosynthetic organisms.

Glucose is broken down by a process known as glycolysis to pyruvic acid which is then decarboxylated to acetate which is then

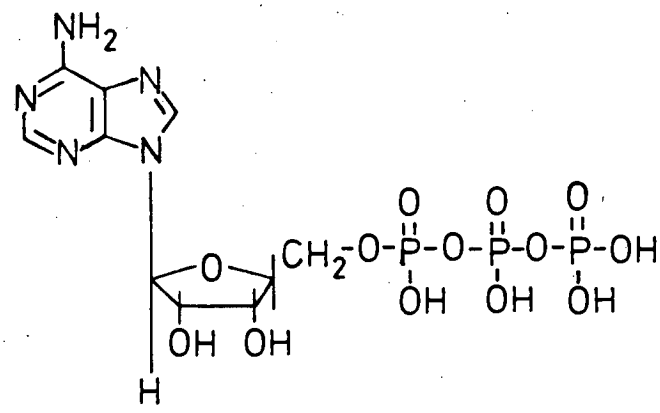
further oxidised to CO_2 by a sequence of reactions known as the tricarboxylic acid cycle.

The pentose phosphate cycle is reversible and as well as forming glucose, can also participate in its breakdown to produce a variety of 3, 4 and 5 carbon fragments of importance, e.g. the 5 carbon sugars or pentoses which are essential constituents of the nucleic acids, and a 4 carbon sugar derivative known as erythrose-4-phosphate which combines with phosphoenol pyruvate, itself a product of both glycolysis and the pentose phosphate cycle to give shikimic acid, the key intermediate in the production of the aromatic amino acids.

Pyruvate, acetate and the intermediates of the tricarbxylic acid cycle together produce the carbon fragments necessary for the biosynthesis of the aliphatic amino acids which with the aromatic amino acids go to produce the proteins, purines and pyrimidines. Acetate is of central importance as it reacts with CO_2 to produce malonate and further reaction of acetate and malonate produces the fatty acids and their derivatives, e.g. prostaglandins. Acetate can also condense with itself to produce acetoacetate and then mevalonate which is the key precursor of the terpenoids. Finally, one of the intermediates of the T.C.A. cycle, succinic acid, is a key precursor to the porphyrins, e.g. chlorophyll, vitamin B_{12} and haem. As indicated in Figure 1, most of the pathways of primary metabolism also give rise to secondary metabolites and the ones we shall be concerned with are those derived from acetate.

1.2 ENZYMES AND CO-FACTORS

The reactions of biosynthesis are all enzyme mediated and each enzyme will catalyse only one particular reaction and will accept only one substrate or at most, a series of structurally similar substrates. Enzymes make use of many co-factors, in the same way that the



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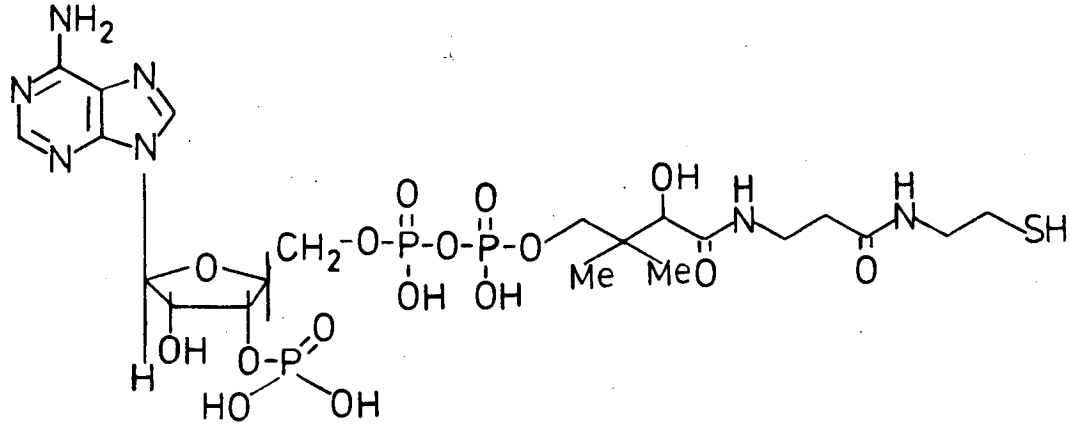

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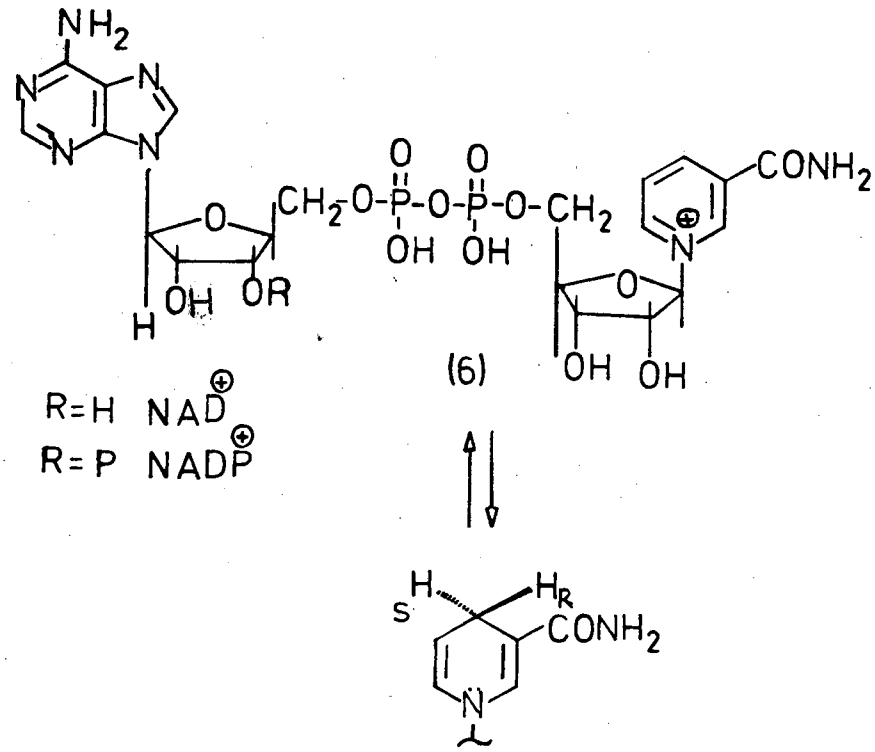
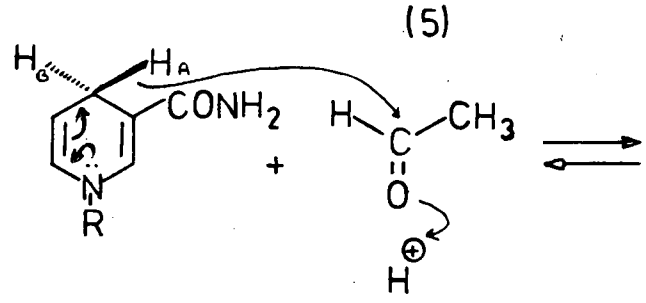
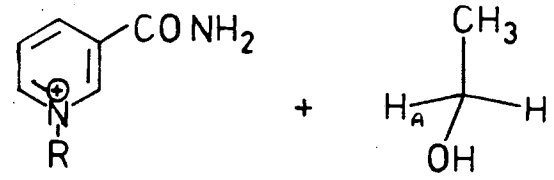

NH2
CH2-O-P-O-P-O-CH2
OH OH
CONH2
OH OR
H
R=H NAD⊕
R=P NADP⊕
(6)
OH OH
H
S
HR
CONH2
N


(5)
HB
HA
CONH2
H
CH3
C
O
N
R
+
H


CONH2
N
R
+
CH3
HA
H
OH

Scheme 1

chemist employs many reagents, and three are of particular importance in all primary and secondary metabolic processes.

1.2.1 Adenosine Triphosphate (A.T.P.)

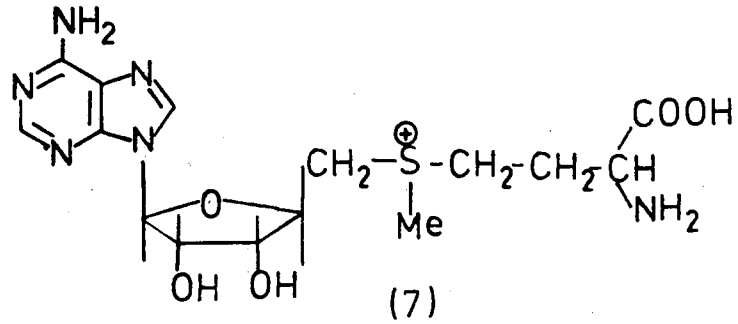
A.T.P. (3) is basically a phosphorylating agent the rest of the structure serving mainly to enable it to fit onto the appropriate enzyme(s) in the correct orientation. Its basic mode of action is to phosphorylate a hydroxyl group to give either the phosphate or pyrophospate. Both of those are excellent leaving groups and so activate the molecule for nucleophilic displacement or elimination. In this way energetically unfavourable reactions can be brought about and the central role of A.T.P. in the energetics of metabolism is endorsed by the fact that the energy liberated on combustion of glucose is stored by conversion of A.D.P. to A.T.P. which then uses this energy to bring about chemical change.

1.2.2 Co-enzyme A (CoASH)

Co-enzyme A(4) is the most important acyl-transfer reagent in living organisms. Despite the apparent complexity of its structure, it is basically a thiol and as such, forms reactive thioesters on reaction with acyl substrates. The thioester activates the acyl species for nucleophilic attack at the ester carbonyl atom with displacement of $CoAS^{\ominus}$ and also for alkylation, i.e. electrophilic attack at the α-carbon atom to bring about *in vivo* analogues of familiar *in vitro* reactions like the Claisen condensation and the numerous alkylation of activated carbonyl compounds.

1.2.3 Nicotinamide Adenine Dinucleotide (NAD^{+}.NADH)

This molecule is the main mediator of biological reductions and oxidations acting as a source of hydride ion in its reduced form (5) and an acceptor of hydride ion in its oxidised form (6). In its reduced form the co-enzyme is prochiral and so the hydride transfer



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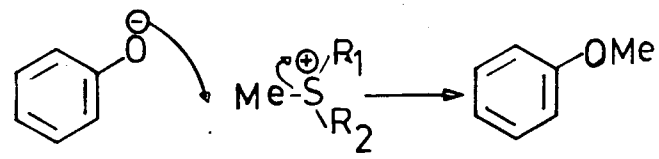
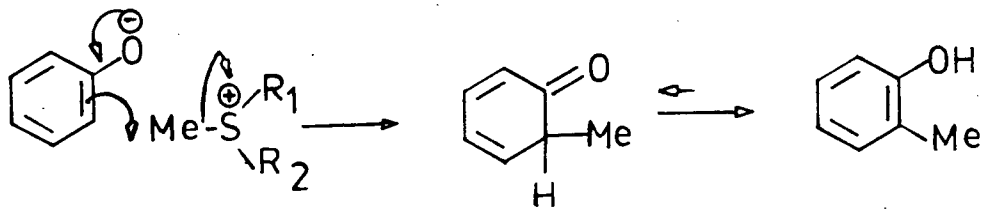
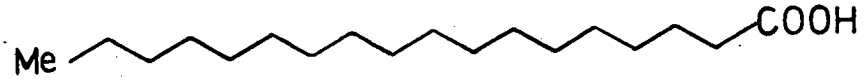




Scheme 2



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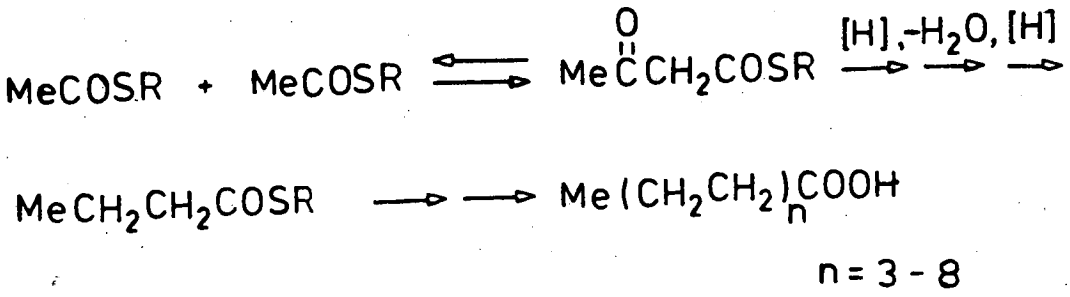
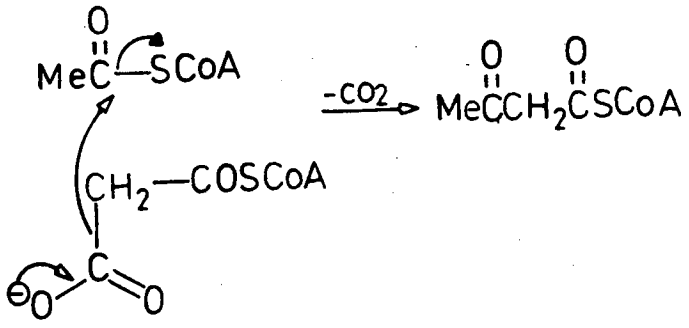
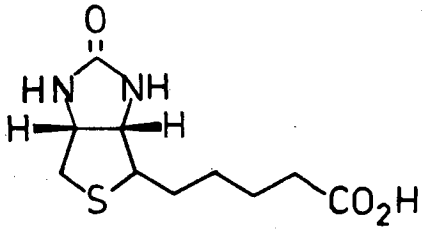


Scheme 3



Scheme 4



(9)

process is usually stereospecific as shown in Scheme 1.

1.2.4 S-adenosyl Methionine (S.A.M.)

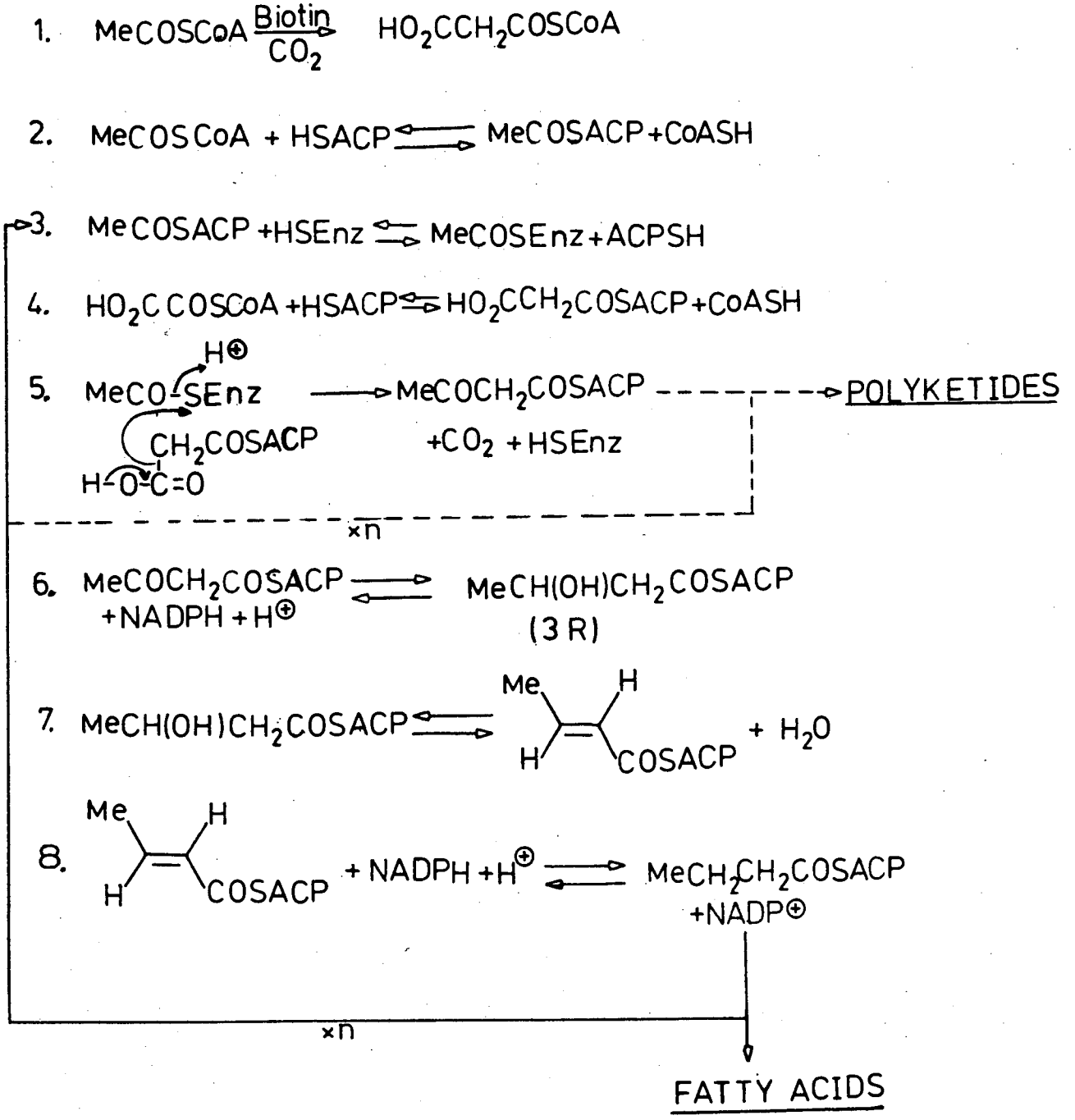
S.A.M.(7) is the co-factor responsible for the introduction of a methyl into metabolites. The process is formally a nucleophilic displacement (Scheme 2).

1.3 FATTY ACIDS

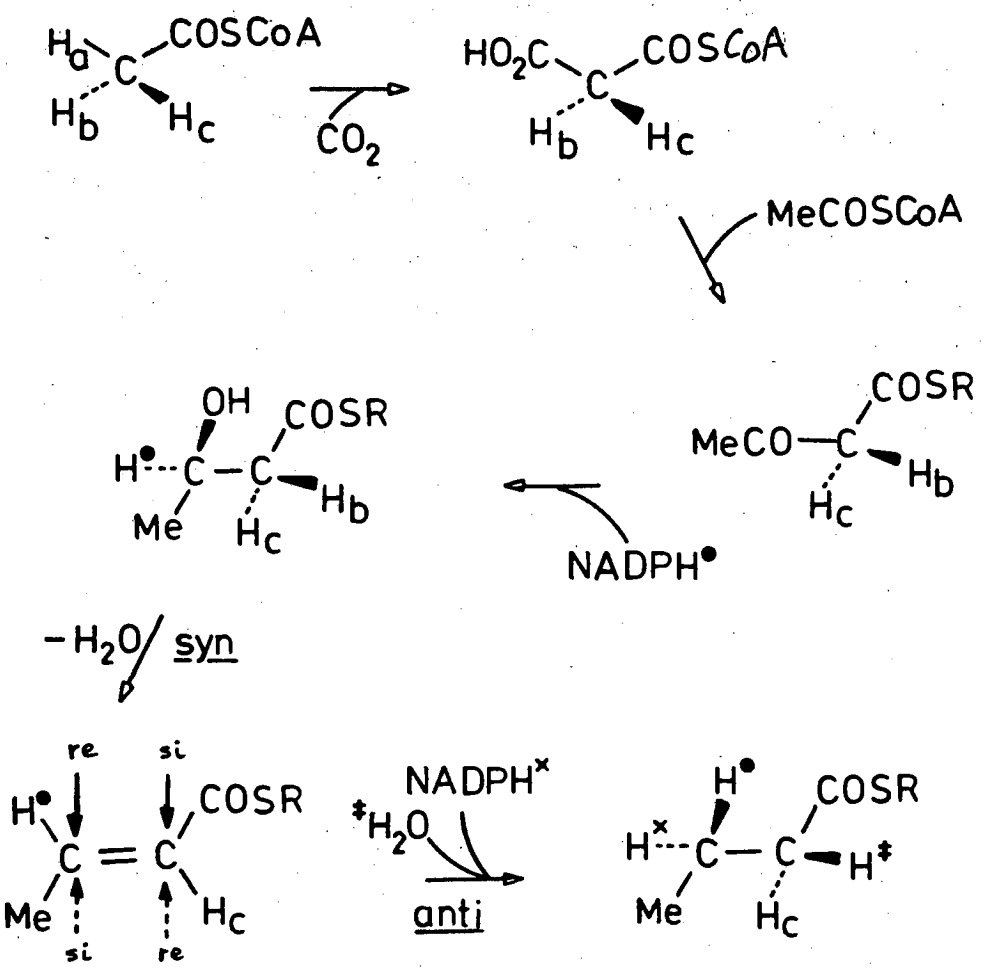
Before talking about polyketides themselves we must first discuss their related primary metabolites the fatty acids.

The common fatty acids comprise a group of straight chain aliphatic carboxylic acids containing even numbers of carbon atoms the most common being stearic acid (8). For many years it was thought that these fatty acids were formed simply by linear combination of acetate units in a series of Claisen condensations followed by reductive loss of oxygen (Scheme 3).[3] However, it was observed by using cell free enzyme system that carbon dioxide was essential for formation of fatty acids from acetate, but if labelled CO_2 was used none of the label ended up in the final product. This observation resulted in the discovery that malonate was involved as the chain extending unit.[4]

Not only is it a better nucleophile than acetate, but the concomitant decarboxylation also helps to drive the reaction in a forward direction (Scheme 4). In the cell acetate and malonate exist as their Co-enzyme A (CoA) thioester derivatives. The importance of thioesters is that they are much more reactive in nucleophilic addition and electrophilic alkylation reactions than their oxygen analogues so that nature by activating these compounds as their thioesters can bring about condensation and related reactions under much milder conditions than are required in the laboratory for similar carbon-carbon bond forming processes. Most of the details of the


1. MeCOSCoA Biotin / CO2 HO2CCH2COSCoA
2. MeCOSCoA + HSACP MeCOSACP + CoASH
3. MeCOSACP + HSEnz MeCOSEnz + ACPSH
4. HO2C COSCoA + HSACP HO2CCH2COSACP + CoASH
5. MeCO-SEnz MeCOCH2COSACP + CO2 + HSEnz
CH2COSACP
H-O-C=O
H⊕
POLYKETIDES
×n
6. MeCOCH2COSACP + NADPH + H⊕ MeCH(OH)CH2COSACP (3R)
7. MeCH(OH)CH2COSACP + H2O
Me
H
COSACP
8. + NADPH + H⊕ MeCH2CH2COSACP + NADP⊕
×n
FATTY ACIDS

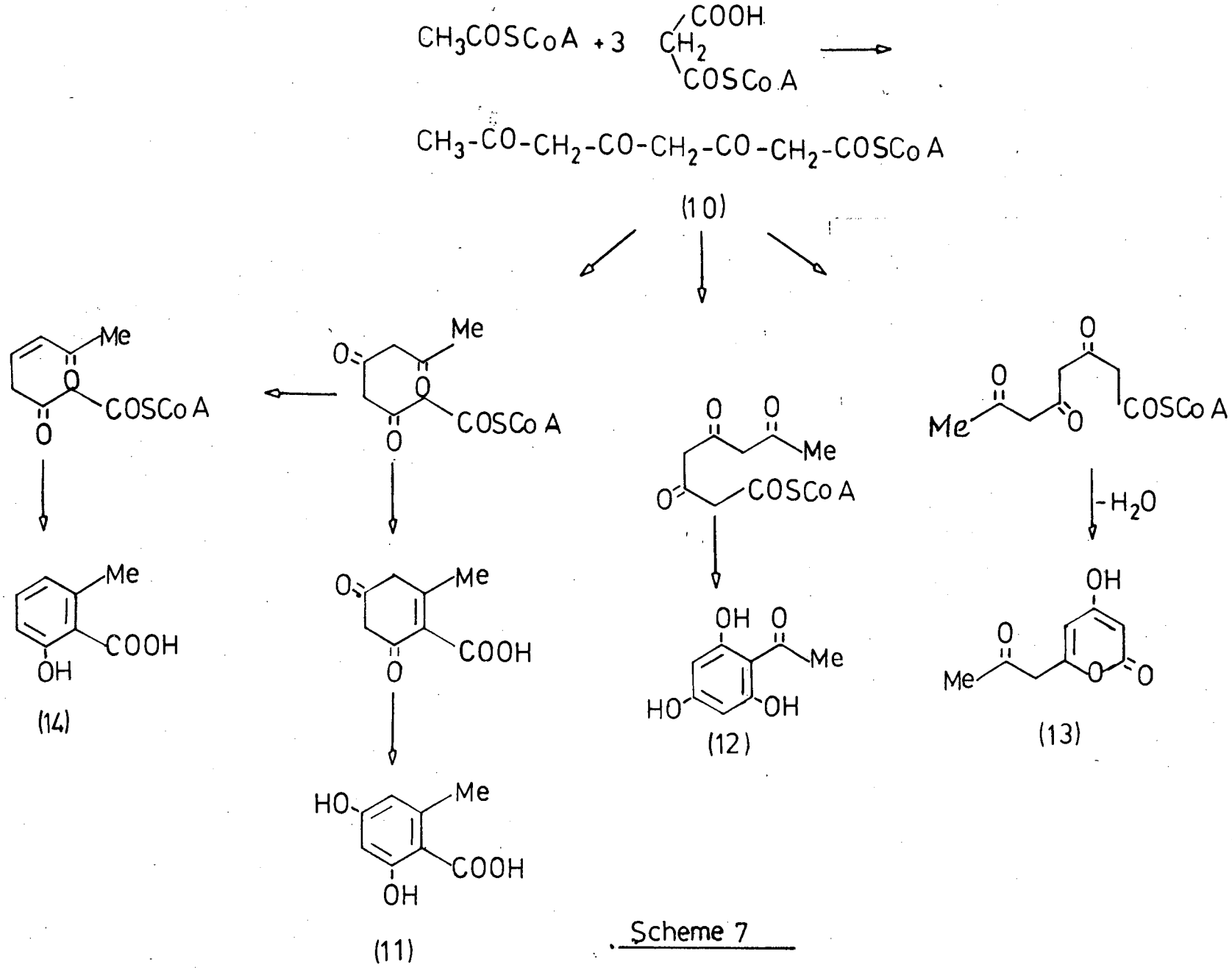
Scheme 5


COSCoA
CO2
HO2C
COSCoA
MeCOSCoA
OH
COSR
Me
NADPH•
COSR
MeCO
-H2O/ syn
re
si
COSR
NADPHx
anti
COSR

Scheme 6

pathway have been elucidated using cell free enzyme systems and purified enzymes, notably by Lynen and Block and is summarised in (Scheme 5).[5]

First acetyl CoA is carboxylated to give malonyl CoA in a reaction mediated by the Co-factor biotin (9).[6] Acetyl CoA and malonyl CoA are then transferred onto the enzyme where they are again attached as thioesters, acetate is then transferred to the enzyme condensing site (again as a thioester) where it condenses with malonate to give enzyme bound acetoacetate which is then reduced by N.A.D.P.H. to give the (3R)-hydroxyacyl intermediate. This then undergoes a facile dehydration to give the unsaturated acyl species which is again reduced by N.A.D.P.H. to the saturated compound. Dehydration of (2R, 3R)-3-hydroxy[2-^{3}H]butyryl thioester by the fatty acid synthetase of yeast has been shown to proceed with retention of tritum to give the trans-2-enoyl derivative, by means of a syn elimination.[7] Assignment of the syn stereochemistry for the dehydration, together with the findings that tritium is retained preferentially from (2S)-[2-^{3}H]malonate, [8] means that the condensation reaction in fatty acid biosynthesis must proceed with inversion of configuration at C-2 of malonate. The stereochemical course of hydrogen transfer in the final reduction has been shown to involve an anti addition of hydrogen via a 2-Si, 3-Si attack on the double bond.[9] Thus the overall stereochemistry of the process is shown in Scheme 6. The saturated acyl species is then transferred back to the condensing enzyme where it can react with a second malonate and so on, the cycle being repeated until the requisite chain length is reached and the fatty acid is released from the enzyme complex known as Fatty Acid Synthetase.[10] The fatty acids themselves give rise to a large number of interesting compounds such as the prostaglandins, polyacetylenes

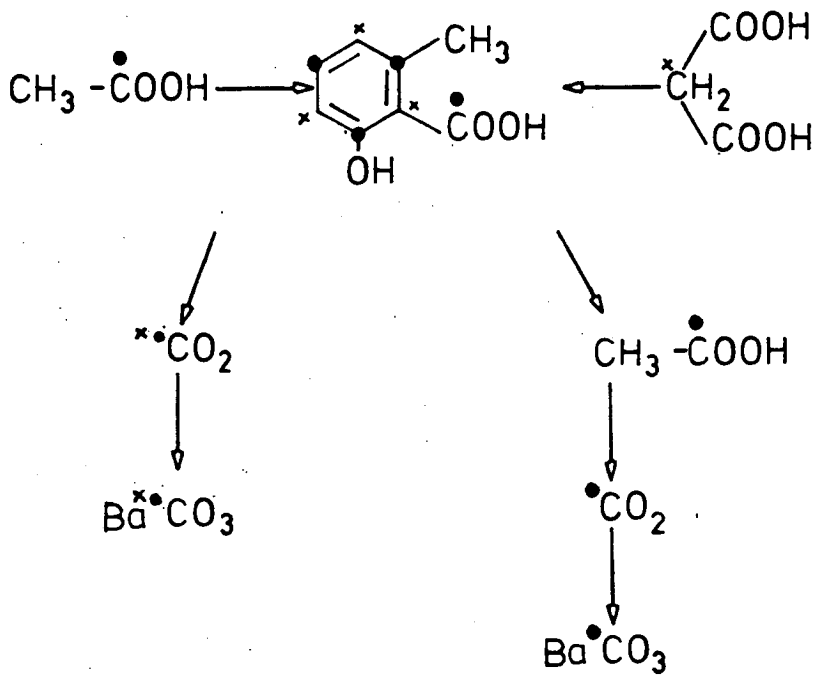

CH3COSCoA + 3
COOH
CH2
COSCoA
CH3-CO-CH2-CO-CH2-CO-CH2-COSCoA
(10)
Me
O
COSCoA
O
(14)
COOH
OH
(11)
HO
(12)
(13)
-H2O

Scheme 7

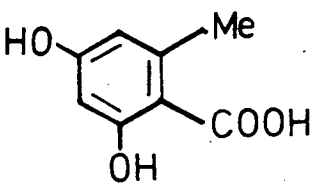
and insect pheromones as well as themselves being integral components of lipids and membrane structures.[11]

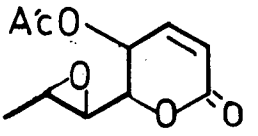
1.4 POLYKETIDES

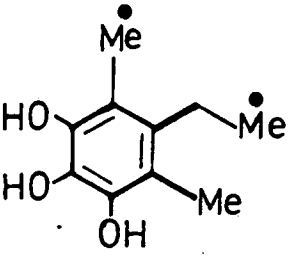
We can now proceed to examine one of the major groups of secondary metabolites; the polyketides, whose biosynthesis is intimately associated with fatty acid biosynthesis. The recognition of the pathway is mainly due to A.J. Birch who observed that many natural products contained aromatic rings with oxygen on every second carbon atom. Birch proposed that these could rise by condensation of acetate units as in fatty acid biosynthesis (Scheme 5), but without reduction of the intermediate β-keto esters to give a β-polyketide further condensation of which would lead to aromatic compounds with the observed oxygenation pattern.[12] He showed that by assuming that various acids RCO_2H could act as the first unit in the β-polyketide chain and that aldol, Claisen condensations and other modifying reactions (see below) might occur, it is possible to rationalise the formation of a large number of natural products.[13] This was soon proved experimentally [14] with the slight modification that the chain extending unit was malonyl CoA as in fatty acid biosynthesis. For example, if one acetate combines with three malonates, the resultant tetraketide (10) can cyclise in a variety of ways to yield aromatic and heterocyclic compounds, e.g. orsellinic acid (11), acetylphloroglucinol (12), and the pyrone (13) as shown in Scheme 7. A slight variation of the orsellinic acid route, by a reduction and elimination process analogous to fatty acid biosynthesis, gives 6-methyl salicylic acid (14) where one of the ketide oxygen atoms has been lost. 6-Methyl salicyclic acid was among the first compounds to be studied by incorporation of labelled precursors. [14] Initially Birch fed sodium [1-^{14}C]acetate. As predicted this led to


CH3 -COOH
CH3
COOH
OH
COOH
CH2
COOH
CO2
Ba CO3
CH3 -COOH
CO2
Ba CO3

Scheme 8







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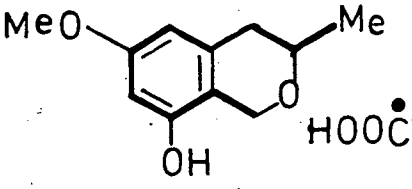

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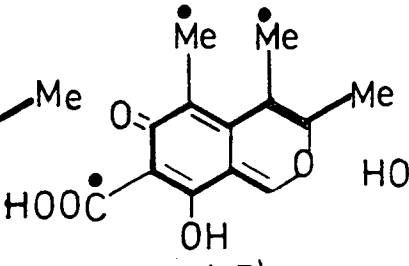

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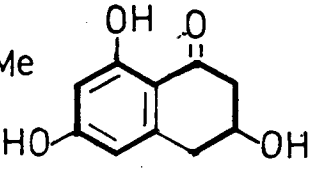

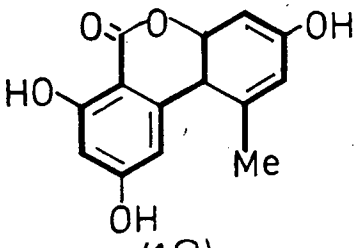

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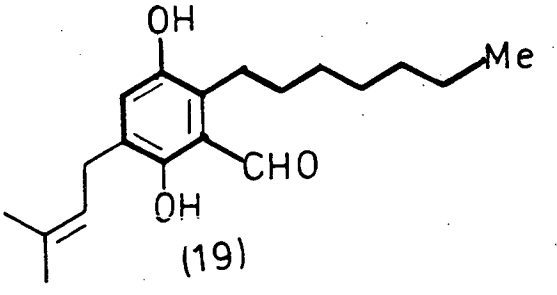

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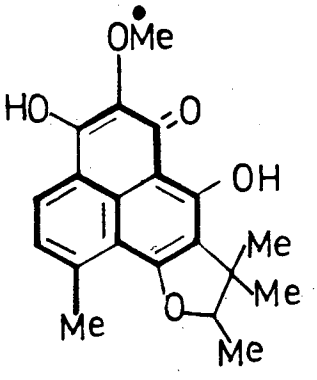

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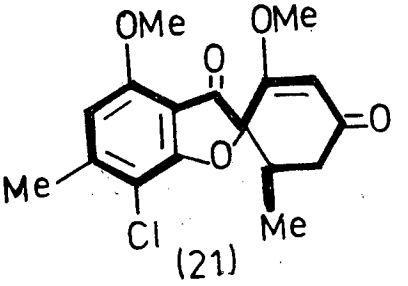
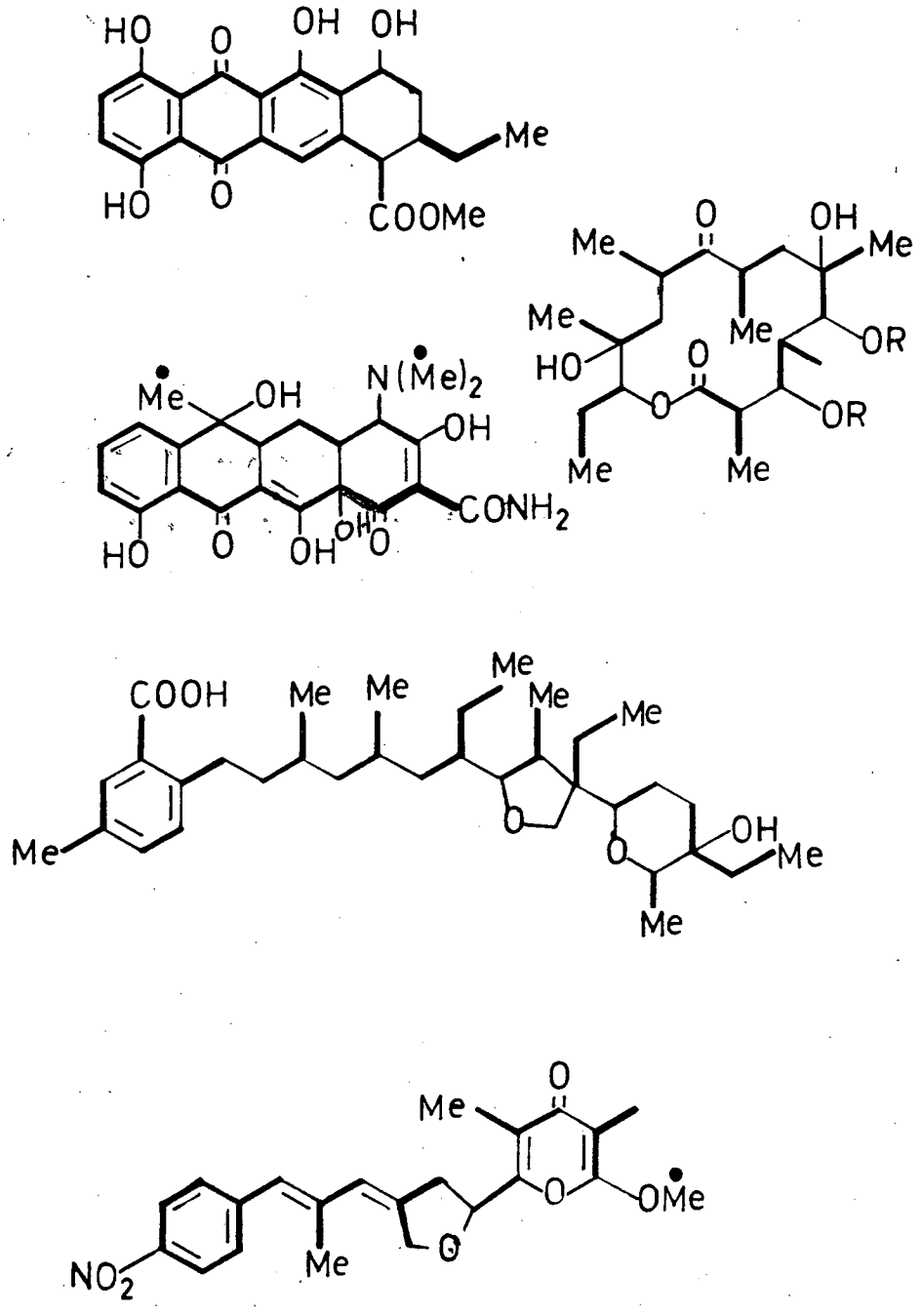


(21)

Scheme 9



Scheme 10

four positions being equally labelled. This was demonstrated by chemical degradation of the molecule to isolate all the labelled carbons separately and determination of their respective radioactivities (Scheme 8). The fact that malonate was an essential precursor of polyketides was shown by incorporation of [1-^{14}C]malonate. Degradation of the resultant 6-M.S.A. showed that only three positions were labelled as expected in contrast to four with [1-^{14}C]acetate. This illustrates a very important principle in biosynthetic studies. It is not sufficient for radioactivity from a labelled precursor to be incorporated into a metabolite. It is essential to show that the label from the precursor labels the final metabolite exactly where and only where the biogenetic theory says it should. This may be achieved by careful synthesis of regio- and/or stereospecifically labelled precursors and careful analysis of the final metabolite to demonstrate the regio and stereospecificity of labelling.

The huge variety of polyketide structures in nature arises from three main variation of the simple pathway we have discussed so far. These are:-

1. Increasing chain length

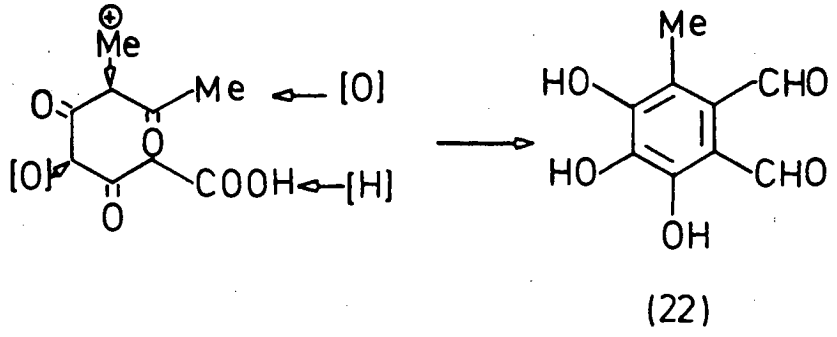
That normally observed is from tetraketides (C_8) to decaketides (C_{20}). Of course as the chain length increases the number of possible ways for the polyketide to coil up and condense increases. Some examples are illustrated in Scheme 9.

2. Alternate "Starter" and "Propagation" Units

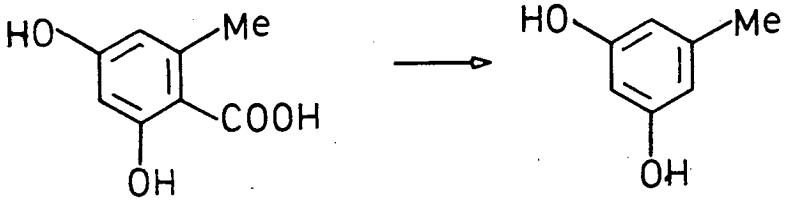
Using different starter acids and different malonate analogues a large variety of structures results (Scheme 10).

3. Modifying Reactions[15]

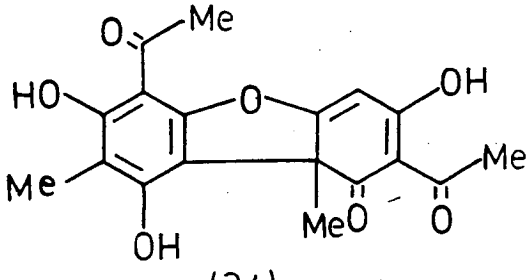
The polyketide chain can be modified by a variety of secondary processes.


Me
Me
[O]
O
O
[O]
COOH
[H]
O
Me
HO
CHO
HO
CHO
OH

(22)


HO
Me
COOH
OH
HO
Me
OH

(11) (23)


O
Me
HO
O
OH
Me
Me
O
O
OH

(24)

These include:-

(a) Loss of one or more oxygens from the polyketide chain as in 6-M.S.A. biosynthesis. In other metabolites the dehydration may be followed by reduction of the resultant double bond (cf. fatty acids) to give partially saturated systems, e.g. flavoglaucin (19).

(b) C and O alkylation with C_1 or C_5 units. We commonly observe the introduction of extra methyls from S-adenosyl-methionine or isoprenoid units from dimethylallyl pyrophosphate. These always occur on nucleophilic (electron rich) sites, e.g. phenolic oxygen or on carbons corresponding to polyketide methylene, e.g. barnol (15), 6-methoxymellein (16), citrinin (17), flavoglaucin (19), and deoxyherqueinone (20).

(c) Electrophilic chlorination, i.e. introduction of Cl at nucleophilic carbon, e.g. griseofulvin (21).

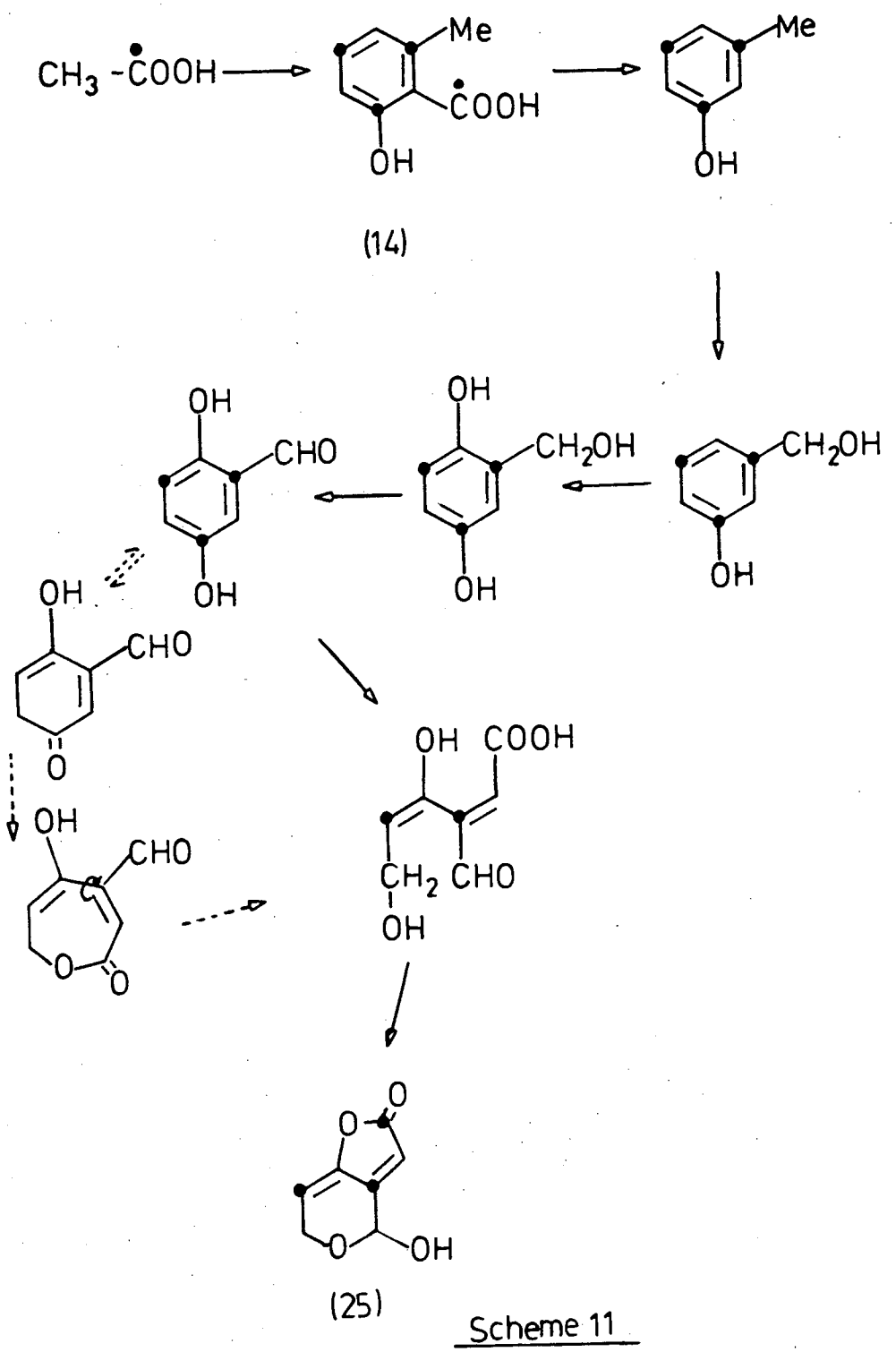
(d) Alkyl groups may be oxidised and carboxyls reduced, e.g. in flavipin (22), an extra methyl group is introduced, the original methyl is oxidised to aldehyde and the original carboxyl is reduced to aldehyde. In fact, we can get full oxidation of methyl to carboxyl and carboxyl reduced to methyl.

$$Me \rightleftarrows CH_2OH \rightleftarrows CHO \rightleftarrows CO_2H \longrightarrow H \text{ or } OH$$

Flavipin (22) also illustrate a further common feature, i.e.

(e) Introduction of extra oxygen atoms, this is probably catalyzed by a mixed function oxygenase.[16]

(f) Decarboxylation, e.g. orcinol (23) is formed by loss of carbon dioxide from orsellinic acid (11). Coupled to the oxidation of methyl to carboxyl, this can result in the loss of a methyl group.


CH3 -COOH
Me
COOH
OH
Me
OH
(14)
OH
CHO
OH
OH
CH2OH
OH
CH2OH
OH
OH
CHO
O
OH
CHO
O
O
OH COOH
CH2 CHO
OH
O
O
O
OH
(25)

Scheme 11

(g) Phenolic oxidative coupling, e.g. griseofulvin (21) and usnic acid (24).

(h) Ring cleavage: More drastic modification of polyketide derived skeleton can occur as a result of oxidative ring cleavage reactions. This happens in the formation of the common fungal metabolite patulin (25) which is shown to be formed from acetate via 6-methyl salicylic acid (14).[18] The sequence of steps has been intensively studied and the sequence established is as in Scheme 11.[19]

Thus in polyketide producing organisms a wide range of secondary processes can occur readily and so result in varying degree of modification, sometimes drastic of the original polyketide derived metabolite.

1.5 TECHNIQUES FOR THE STUDY OF POLYKETIDE BIOSYNTHESIS

As in all biosynthetic studies, there are two basic questions to be answered:

(a) What is the nature of the molecular building blocks? and

(b) What are the intermediate steps between the building blocks and the final product?

The structure of a polyketide often allows accurate speculation about its origins and even its mechanism of formation. This is based on the fact that large numbers of polyketides are formed from one or two simple repeating units. Also identification of metabolites of similar structures in the same or related species is of great help. (See the arugosins and variecoxanthones in Chapter 2). To track the likely precursor through to the metabolite, the precursor must be labelled or marked in some way. The next step is to feed the micro-organism growing in liquid culture with a labelled precursor. Commonly aqueous

solutions are added at predetermined time(s) to the culture and the metabolite is isolated and purified some time later. Common problems that can arise during feeding experiments are:-[20]

(i) The precursor may be utilized in metabolite biosynthesis at very low level or not at all. This may be (a), because it is genuinely not involved in the biosynthesis of the chosen metabolite or (b), because of the difficulty in getting the precursor to the site of biosynthesis or, (c), because it is used much more efficiently for the formation of another secondary or primary metabolite. Thus a negative result with a labelled precursor is always ambiguous, never certain.

(ii) Even if a labelled compound is shown to be an efficient precursor for a metabolite D (eq. 1.1), it does not mean it is an obligatory intermediate in the biosynthesis of D.

$$\begin{array}{ccccccc} A & \longrightarrow & B & \longrightarrow & C & \longrightarrow & D \\ & & \uparrow & & & & \\ & & E & & & & \end{array} \qquad (1.1)$$

It may just have been a convenient foreign substrate dealt with economically by the living system. However, if the precursor C can be shown to occur in the organism under examination and be formed like the metabolite, D, from the same earlier precursor(s) e.g. A, then it is most likely to be a true intermediate.

(iii) The precursor may be at a shunt E from the main pathway in equation (1.1). It is difficult to resolve whether or not this is so and final conclusions depend on the balance of evidence.

A variety of labels can be used:

1.5.1 Radioactive Isotopes[21]

^{14}C with a half life of 5600 years is the most commonly used radioactive isotope. It is used extensively to monitor the uptake of precursor(s) into metabolites and to determine the fates of individual carbons in a precursor during biosynthesis. It is known not to be lost during biosynthesis. The isotope ^{14}C is a β-emitter and is assayed by liquid scintillation counting. In simple terms, the method involves dissolving the radioactive compound in a scintillant "cocktail" containing phosphors which convert the β-Radiation into light. The light emitted is measured within the scintillation counter by photomultiplier tubes and is expressed as counts against time, a normal way of expressing radioactivity. In practice radioactivity is measured within the counter at less than 100% efficiency. The actual efficiency must be determined and allowance made for this in calculating the true activity of the sample (in disintegrations sec^{-1} m mol^{-1}; 1 curie = 3.7×10^{10} d sec^{-1}). Reliable radioactivity measurements depend:-

(i) On recrystallization of the sample or a derivative, to constant radioactivity, and

(ii) On obtaining counts for the radioactive compound at at least twice the value for an inactive background sample (this is the lower limit for radioactivity measurement).

The fate of radioactive carbons is determined by degradation of the radioactive metabolite to isolate the labelling site from all others (See Scheme 8). Specific labelling of a particular site or sites indicates specific utilisation of the precursor. The ^{14}C technique is highly sensitive which lowers the amount of ^{14}C labelled substrates necessary for labelling studies, so they are almost always fed in

trace amounts with minimum disturbance of normal metabolism. If accurate quantitative data on the incorporation of labelled atoms into a product is needed, but precise location of the label is unimportant, use of ^{14}C labelling studies is indicated, and it is the method of choice to establish the most efficient feeding regimen prior to ^{13}C feeding studies (see below). ^{14}C - labelling studies are less attractive when it is necessary to know the exact location of the label within a metabolite for not only must the compound be radiochemically pure but chemical manipulation to determine enrichment at its various centres are frequently time consuming and conducive to cumulative errors.

1.5.2 Stable Isotopes

The most important stable isotopes in biosynthetic studies of polyketides are ^{13}C, ^{2}H and ^{18}O.

The advent of ^{13}C n.m.r.[22] spectroscopy provides a non-destructive detection system, capable of determining the location and relative concentration of each chemically nonequivalent carbon in a molecule. Since the ^{13}C nucleus has a nuclear Spin I = $^{1}/2$, nuclear magnetic resonance signals may be observed. With the development of sensitive instruments and the means to accumulate (and thus enhance) weak signals, the amount of data available on ^{13}C chemical shifts and coupling constants (JC-C JC-H) is increasing rapidly.[24] The undecoupled ^{13}C n.m.r. spectrum shows spin-spin coupling to directly-bonded hydrogens and smaller splitting due to hydrogens on adjacent carbons and this is often of some utility in elucidation of structure,[24] but the spectra are usually proton-decoupled so that a single line is found for each carbon in the molecule. Thus, although the natural abundance of ^{13}C is only 1.1 percent, it is now relatively easy to obtain ^{13}C spectra for any compound given sufficient material

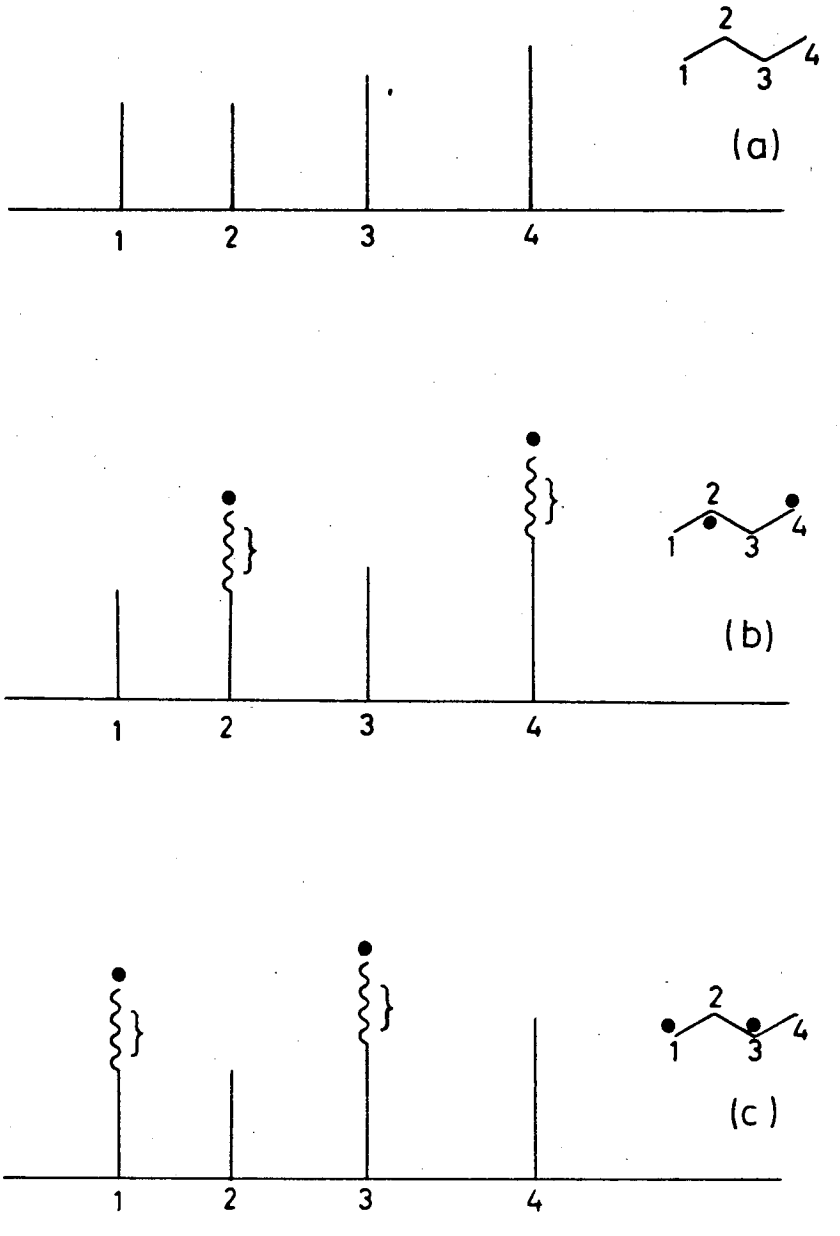


Figure 2 Simulated proton noise-decoupled ^{13}C n.m.r. spectra of a hypothetical polyketide derived molecule (a) at natural abundance, (b) enriched from [1-^{13}C]acetate and (c) enriched from [2-^{13}C]acetate.

(ca 50mg). One great advantage of ^{13}C n.m.r. is the considerable spread of chemical shifts; about 200 p.p.m. compared with about 15 p.p.m. for ^{1}H shifts. These shifts are in the same relative order as proton shifts, i.e. alkyl carbons have shifts at higher field (smaller value) than aromatic carbons and carbons carrying oxygen substitutents have larger values than those bearing carbon or hydrogen substitutents, the carbonyl carbon usually provides the lowest field signal.

1.5.3 ^{13}C - Enrichment Studies:[25]

If we consider any four contiguous carbons in a polyketide derived molecule, normally these four carbons will be derived from endogenous acetyl CoA produced by the cell's normal metabolism and will contain only natural abundance ^{13}C (1.1%) and so in the p.n.d. ^{13}C n.m.r. spectrum each carbon will give rise to one sharp line (of more or less equal intensity) (Figure 2a). Now if we add sodium acetate in which the carboxyl carbon is highly enriched (ca 95%) with ^{13}C, ([1-^{13}C]acetate) then this exogenous acetate will be diluted to a greater or lesser extent by the endogenous acetate pool but some of it will be incorporated into the metabolite and so those carbons, say C-2 and C-4, which were originally derived from the carboxyl carbon of acetate will contain extra ^{13}C and this extra ^{13}C will manifest itself as an increase in the appropriate signal intensities in the proton noise decoupled ^{13}C spectrum of the enriched metabolite (Figure 2b). If we now add acetate in which the methyl carbon is enriched ([2-^{13}C]acetate) then we shall see enrichment of the alternate signals (Figure 2c). Thus simply by feeding ^{13}C labelled precursor and determining the ^{13}C n.m.r. spectrum the signals which show enhanced intensities give us the site of enrichment, provided we can assign the ^{13}C spectrum unambiguously. Note that at this stage the ^{13}C labelling

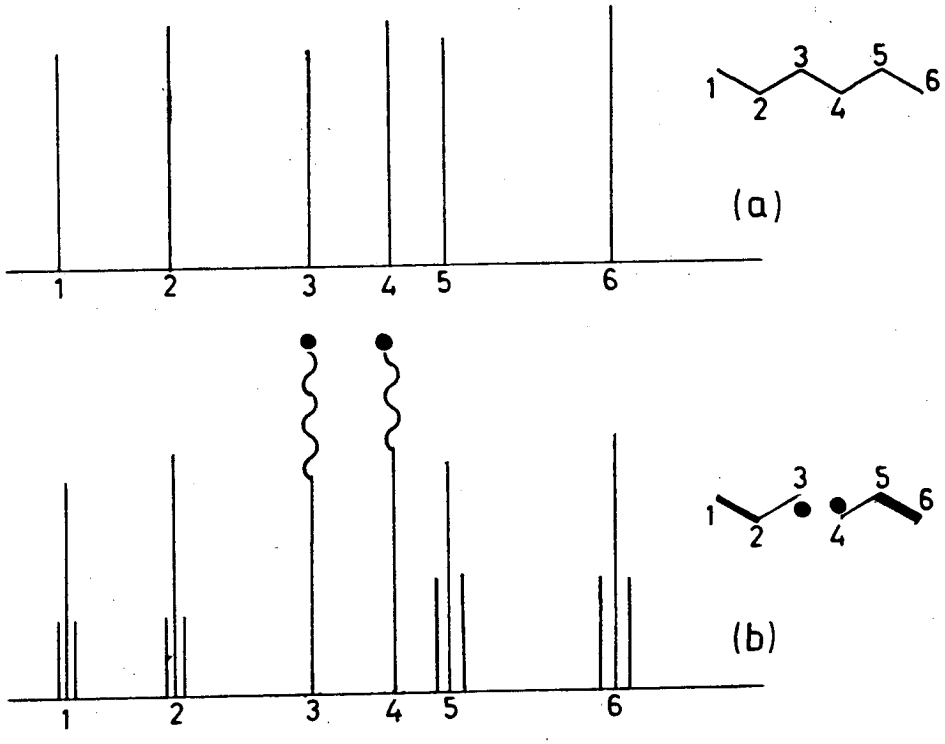


Figure 3 Simulated p.n.d. ^{13}C n.m.r. spectra of a polyketide derived molecule (a) at natural abundance and (b) enriched from [1,2-$^{13}C_2$]acetate.

method does not give us any information which could not (in theory at least) have been obtained by classical ^{14}C techniques, though in practical terms it is much more comprehensive.

The real advantage of ^{13}C methods comes when precursors doubly labelled with ^{13}C are used. If we now consider a molecule of acetate in which both carbons are entirely ^{13}C, ([1,2-$^{13}C_2$]acetate) then we have two adjacent nuclei of spin $^1/2$ and so they will couple to each other (in the same way as adjacent protons). If this acetate molecule is incorporated intact into a metabolite then in any individual molecule these pairs of carbons derived from an orignally intact acetate unit must necessarily both be enriched simultaneously and so will show a mutual ^{13}C- ^{13}C coupling. Thus if C-1 is enriched then C-2 must necessarily also be enriched. What we see in the resultant ^{13}C n.m.r. spectrum is the natural abundance signal flanked by ^{13}C-^{13}C coupling satellites (Figure 3b). By analysing the coupling patterns we can obtain information on the way in which acetate molecules are assembled on the enzyme surface and on the way the precursor polyketide chain folds up prior to condensation and cyclisation. This is information which would be very difficult if not impossible to obtain by classical ^{14}C labelling methods. We can take a step further because if at any stage in the biosythesis the bond joining two carbons derived originally from an intact acetate unite is broken, then the ^{13}C- ^{13}C coupling is lost and these carbons then appear simply as enriched singlets thus in this way we can detect bond cleavage and rearrangement processes occurring during biosynthesis. Figure 3b shows how the original bond between C-3 and C-4 is broken and so the coupling is lost.

1.5.4 Deuterium

Deuterium is a stable isotope readily available at a high level

of enrichment. It is a suitable substitute for protium since the properties of deuterium labelled compounds mirror closely those of protio-compounds. Until recently [26] it has been detected routinely by mass spectroscopy, a method which is statistical and of low sensitivity and its only advantage is that evidence for sites of labelling can be obtained without recourse to degradative experiments. By using ^{1}H n.m.r. the presence of ^{2}H in a biosynthesized molecule could be detected by the deletion [27] of a specific absorption which appears in the spectrum of the analogous unlabelled compound. Sometimes this method has been used in conjunction with mass spectrometry to measure the number of deuterium atoms incorporated as well as its location, provided that care is taken to ensure that exchange with protons in the medium does not occur.

1.5.5 Deuterium n.m.r.[28]

Deuterium is a quadrupole nucleus with spin 1. As a consequence of the lower magnetogyric ratio (0.154) deuterium resonates at 15MHz in a magnetic field of 23KG compared with 100MHz for ^{1}H. The chemical shift values obtained in deuterium n.m.r. spectra are closely similar to those of the equivalent protons, although the scale in Hz is only 15% that of the proton n.m.r. Coupling constants $J(^{2}H - ^{1}H)$ also approximate to one-sixth the value of $J(^{1}H - ^{1}H)$. The relaxation behaviour of deuterium is dominated by a quadrupole exchange mechanism and this results in extensive line broadening which in turn causes poor resolution. The main advantage of using ^{2}H n.m.r. are:[29]

(a) It is a relatively inexpensive isotope.

(b) The absence of contamination dangers, mean that does not require special handling.

(c) Its low natural abundance (0.016%) enables the incorporation of deuterium labelled precursors to be positively identified more easily.

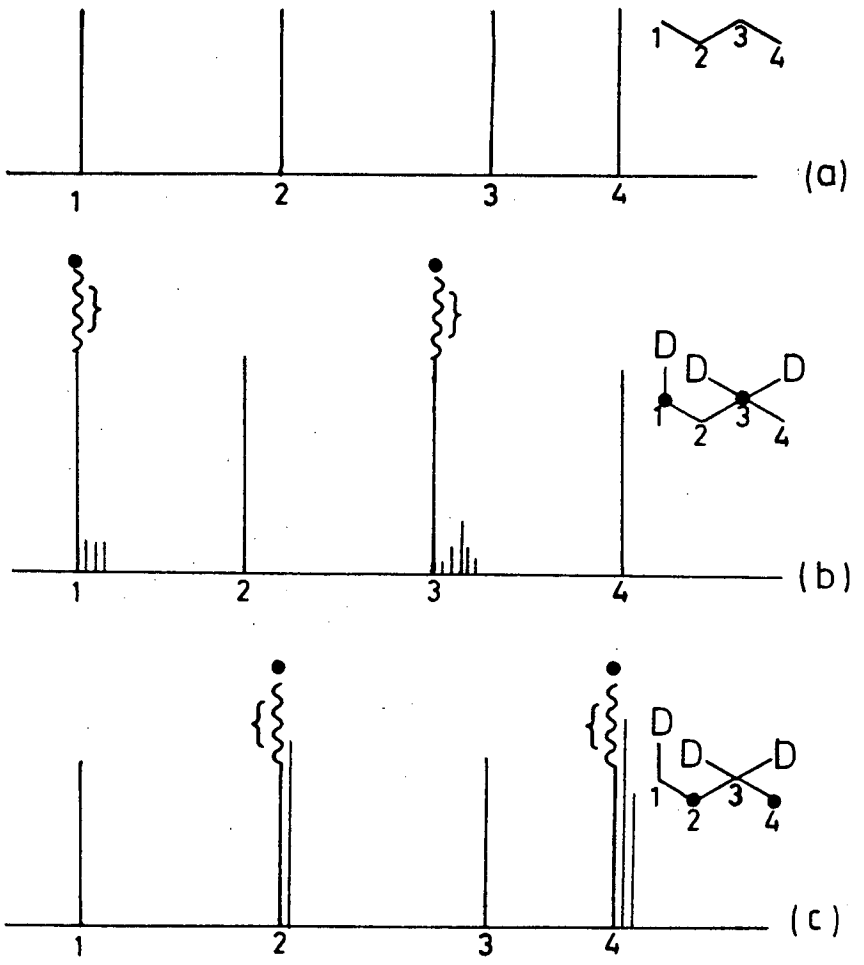
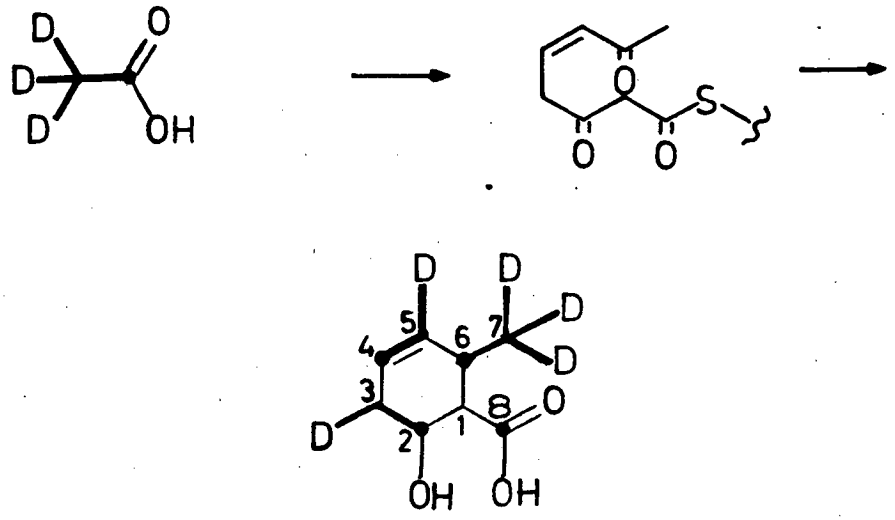


Figure 4 Simulated p.n.d. ^{13}C n.m.r. spectra of a polyketide derived molecule (a) at natural abundance, (b) enriched from [2-^{13}C, $^{2}H_3$]acetate and (c) enriched from [1-^{13}C, $^{2}H_3$]acetate.

1.5.6 Indirect Methods for Detection of ^{2}H Incorporation

(i) α - Isotope Shifts ^{2}H induces an upfield shift [30] in the resonance of the ^{13}C nucleus to which it is directly attached. The signal appears as a triplet whose lines are of equal intensity, and is centred 0.3-0.6 p.p.m. upfield of the normal protonated ^{13}C signal. The presence of each additional deuterium shifts the signal a further 0.3-0.6 p.p.m. upfield and increases the multiplicity; thus, $-CD_2-$ appears as a quintet with signals of intensity (1:2:3:2:1) (Figure 4b) and a CD_3 as a septet (1:3:6:9:6:3:1). The different signals for a methyl group overlap to a certain extent and are also superimposed upon the normal signal from protonated nuclei. A partially deuteriated methyl thus gives rise to a complex signal comprising lines from $-CD_3$ - CD_2H - CDH_2 - as well as CH_3 labelled species.[31] Fortunately, the spectral analysis can be simplified by determining the spectrum with deuterium decoupling instead of proton decoupling,[32] interference from protonated nuclei is greatly reduced in this mode of operation because of the loss of n.O.e. Recent spectral editing techniques[33,34] are also a feasible aid for the detection and assignment of isotopically shifted ^{13}C signals in deuteriated molecules at the level of isotopic enrichment attainable in a typical biosynthetic experiment. In polyketide biosynthesis, use of deuterium in conjunction with ^{13}C has proved fruitful. Although much of the hydrogen label is sometimes lost by exchange from the activated methylenes of the polyketide chain, the possibility remains that more than one isotopic label may be retained at positions derived from the methyl group of acetate. The number of deuteriums detected at a given site can be used to identify the chain starter unit[35] or to distinguish between possible biosynthetic intermediates, thereby yielding information about the mode of biosynthesis. The extent of



(14)

Scheme 12

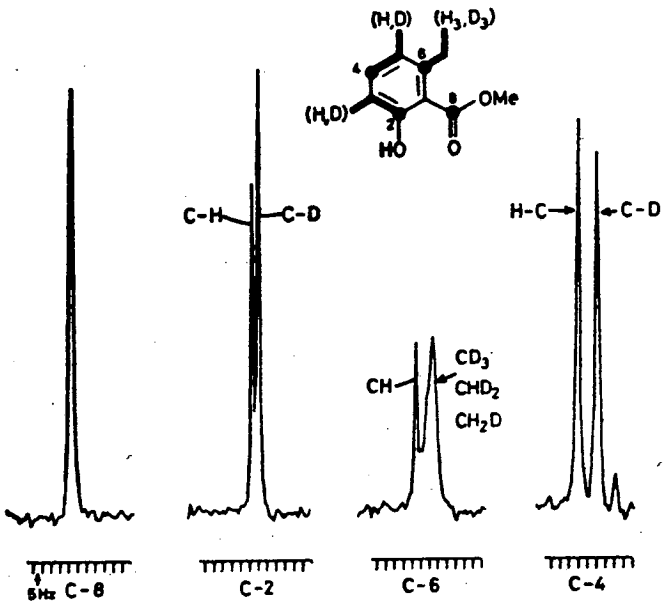
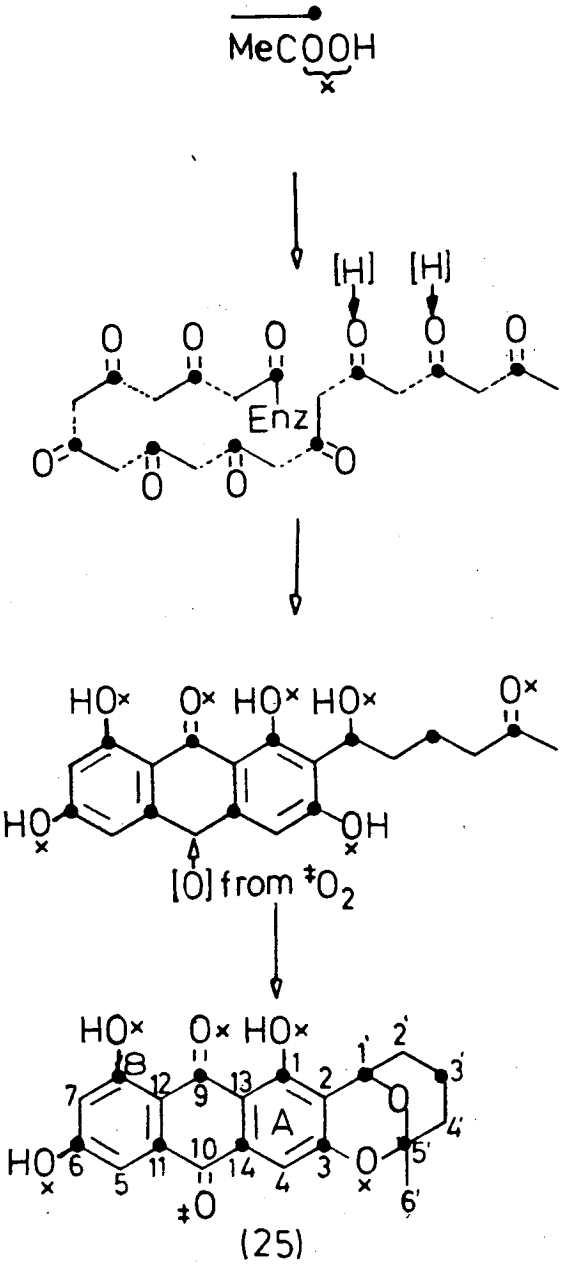


Figure 5 Signals from the p.n.d. ^{13}C n.m.r. spectrum of 6-methylsalicylic (14) derived from [1-^{13}C, $^{2}H_3$]acetate.[36]

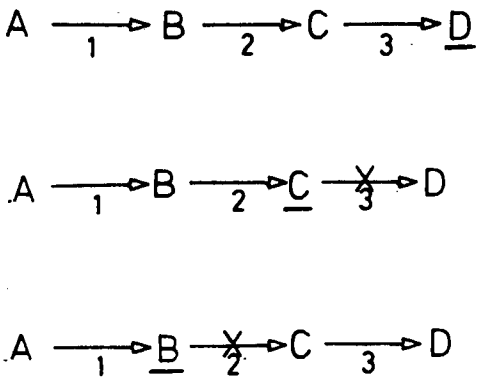
deuterium retention may be correlated to the amount of hydrogen exchange during biosynthesis and may thus provide clues to the complex mechanisms involved in the building up of the carbon skeleton. This technique provides a useful alternative to the standard procedure of using ^{13}C-labelled malonate to pick out the chain starter unit.

(ii) **β-Isotope Shift Method** [36]

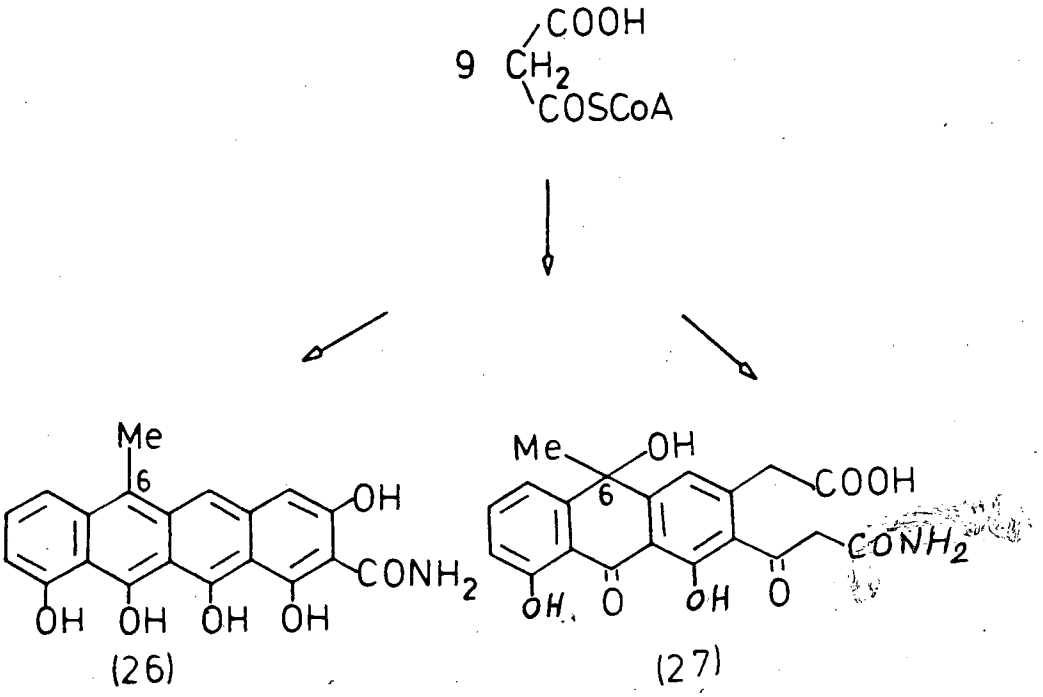
Although the indirect method of monitoring deuterium which was discussed in last Section (α- shift method) has proved quite an attractive one, for quantitative measurement of deuterium enrichment it is not the ideal method, because the α- shift method requires simultaneous ^{1}H and ^{2}H noise decoupling to be satisfactory and these facilities are not readily available on most spectrometers. This problem can be partly overcome by placing the reporter ^{13}C nucleus β to the deuterium. Here again the presence of deuterium is detected by an upfield shift (β-shift) in the resonance of ^{13}C nucleus. But the ^{2}H- ^{13}C coupling over two bond is negligible (<1 Hz) so the shifted signal is effectively a singlet in the proton noise decoupled ^{13}C n.m.r. spectrum. In this case the shifted signal is centred Ca 0.05 p.p.m. upfield of the normal protonated signal. Evidently the presence of each additional deuterium shifts the signal a further 0.05 p.p.m. upfield as shown in Figure 4c. As it can be seen it is more easily determined because the problems with relaxation and n.O.e. are avoided. This technique has been used for a quantitative study of deuterium exchange in the biosynthesis of polyketide 6-methylsalicylic acid (14) from [1-^{13}C, $^{2}H_3$]acetate.[36] The molecule is formed from four C_2 units via a linear polyketide (Scheme 12). The p.n.d. ^{13}C n.m.r. spectrum (Figure 5) clearly shows that upfield β-shifted resonances were observed for C-2, C-4 and C-6 but not for C-8.



Scheme 13



Scheme 14



Scheme 15

1.5.7 Use of ^{18}O in Biosynthetic Investigations:

^{18}O with a natural abundance of 0.2% is another stable isotope which has long been used to trace the fate of oxygen in biosynthetic transformations. Until quite recently mass spectrometry was the only way to determine its presence. This method mostly shows the amount of incorporated ^{18}O but not the position of the label(s). It has recently been shown that ^{18}O induces an upfield isotope shift in ^{13}C n.m.r. spectra if it is directly attached to the nucleus.[37] This new non-degradative technique allows semi-quantitative localization of ^{18}O in highly oxygenated systems where extensive degradations and preliminary labelling would be necessary to determine structures of mass spectrometric fragments. This method has been used to show that the incorporation of $^{18}O_2$ gas and doubly labelled sodium [1-^{13}C, $^{18}O_2$] acetate into averufin (25) occurs as indicated in Scheme 13.[38]

1.5.8 The Use of Mutants in Biosynthetic Studies[39]

After successful preliminary experiments with ^{14}C, ^{13}C, ^{2}H, ^{18}O labelled precursors suggest a reasonable biosynthetic pathway for the biosynthesis of a natural product, further support for the proposed biosynthetic pathway could be achieved by using mutant strains of the original organism. For example if the sequence of the biosynthesis of a metabolite D is as in scheme 14 where A, B and C are known intermediates, blocking step 2 or 3 will produce mutant strains that only accumulate B or C respectively. The identification of the structures of these intermediates would then throw light on the biosynthetic pathway by which D is being produced. Mutation can be accomplished by ultraviolet radiation or by treatment with mutagens. This method has been usefully applied to antibiotics of the tetracycline series.[40] It can be seen in Scheme 15 the assembly of

molecules of malonyl CoA followed by reduction and cyclisation gives rise to 6-methyl-pretetramid (26), but it was not clear whether C-methylation occurred after or before cyclisation. Impairment of the cyclisation step (by mutation) without affecting the nature of the assembly, produced the partly cyclized intermediate (27) whose structure clearly shows that C-methylation occurs prior to cyclisation. However, this method suffers some disadvantages:

(a) Mutants will normally continue to grow alongside the non-mutant parent stock and "screening" for their existance is tedious and time consuming.

(b) Enzymatic reactions in secondary metabolism are less specific so several parallel reactions of different substrates may be blocked by a single mutation.

(c) Compounds (intermediates) formed as the result of mutation may still be substrates for the remaining enzymes, so that the products finally accumulating are highly modified and the position of the defective step in the sequence is not easily elucidated.

1.5.9 Biosynthesis in Cell Free Systems

All of the biosynthetic processes that are mentioned in this introduction are based on methods which are more or less indirect. Powerful support for any biosynthetic pathway as well as detailed information on the reactions involved may be gained by isolation, purification, and characterization of enzymes which will catalyse individual steps of biosynthesis. Experiments with cell free systems have shown that the active acetyl and malonyl units of polyketide biosynthesis are the coenzyme A derivatives.[41] But it has not yet proved possible to isolate a pure enzyme capable of polyketide biosynthesis. Cell free extracts capable of synthesizing orsellinic

acid (11)[42], 6-methylsalicylic acid (14)[43] and alternariol (18)[44] have been prepared and partial purification of the enzymes concerned has been achieved. In each case there is evidence for a multi-enzyme complex. But so far, this method has found only limited application in the study of secondary biosynthetic systems. The reasons for this are not readily apparent, but it is possible that these systems require integrated associations of enzymes, perhaps connected with structural elements in the intact-cell which are particularly sensitive to disruption.

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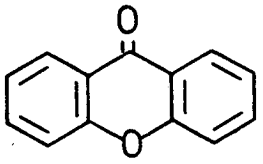
CHAPTER 2

STUDIES RELATING TO THE BIOSYNTHESIS OF TAJIXANTHONE



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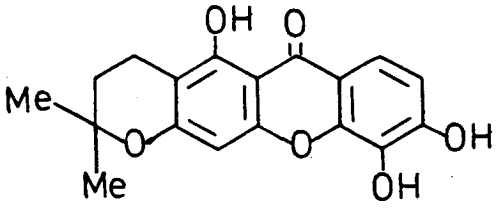

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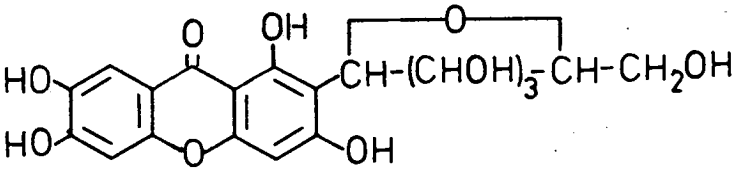

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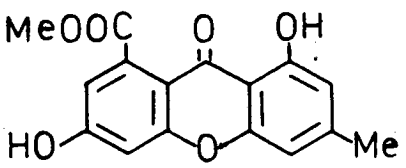

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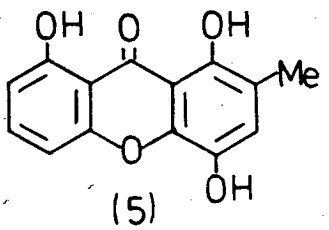

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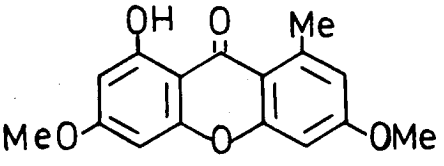
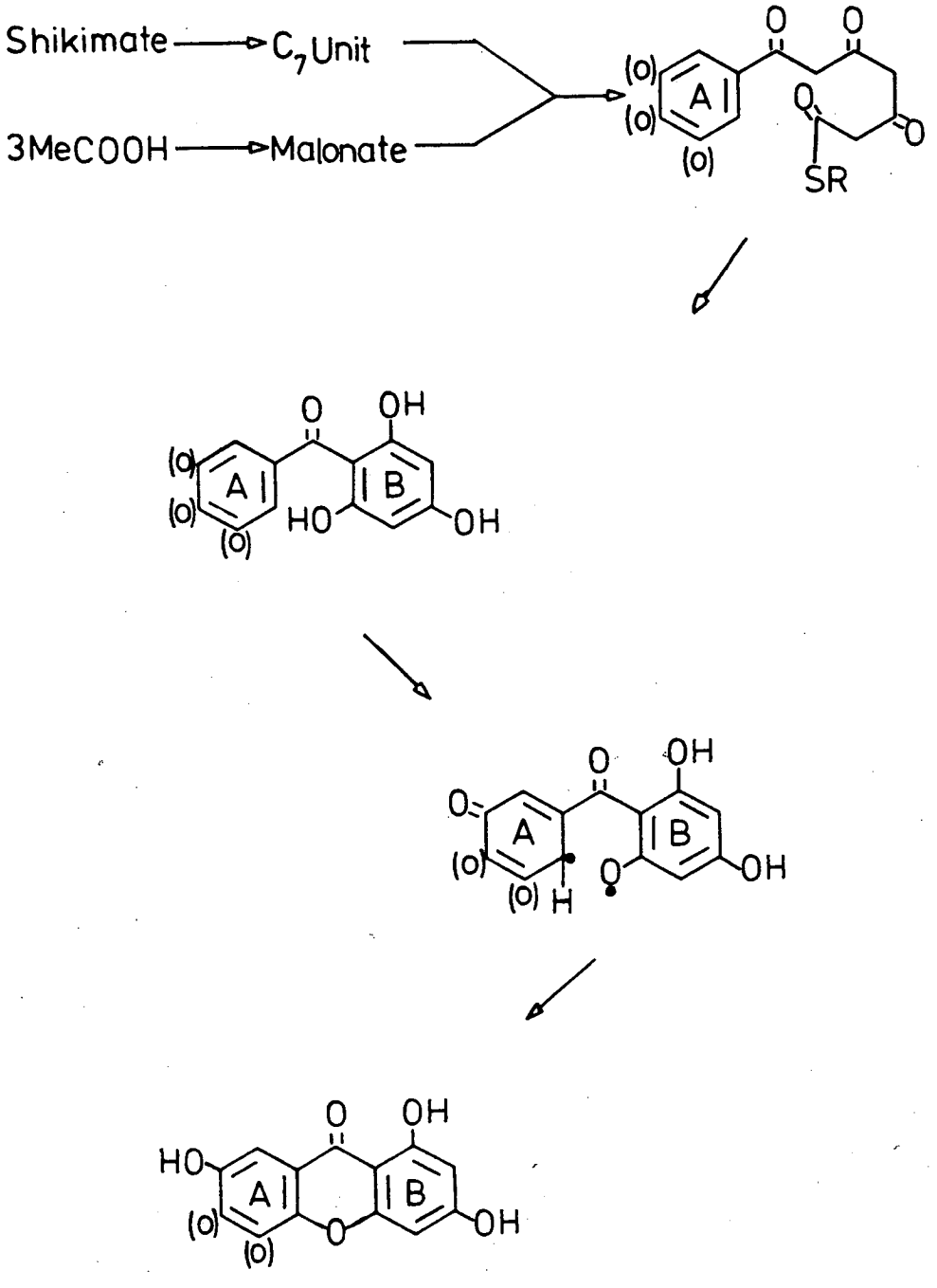


(6)

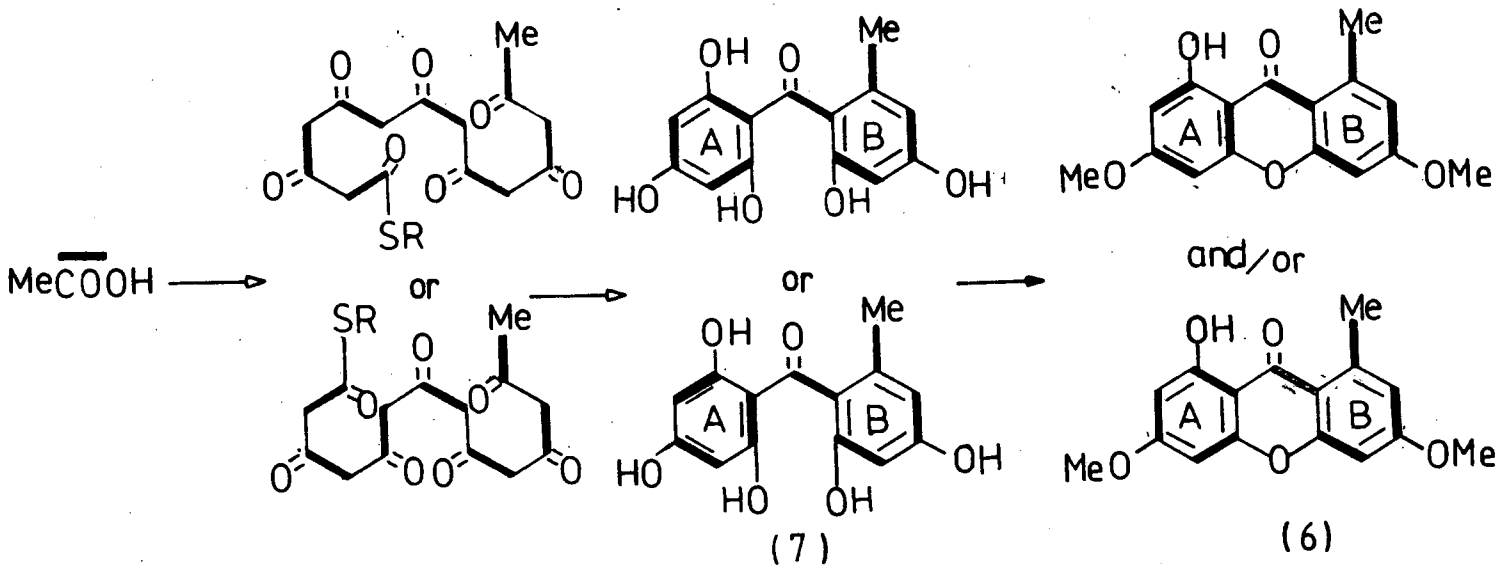
2. STUDIES RELATING TO THE BIOSYNTHESIS OF TAJIXANTHONE

2.1 INTRODUCTION

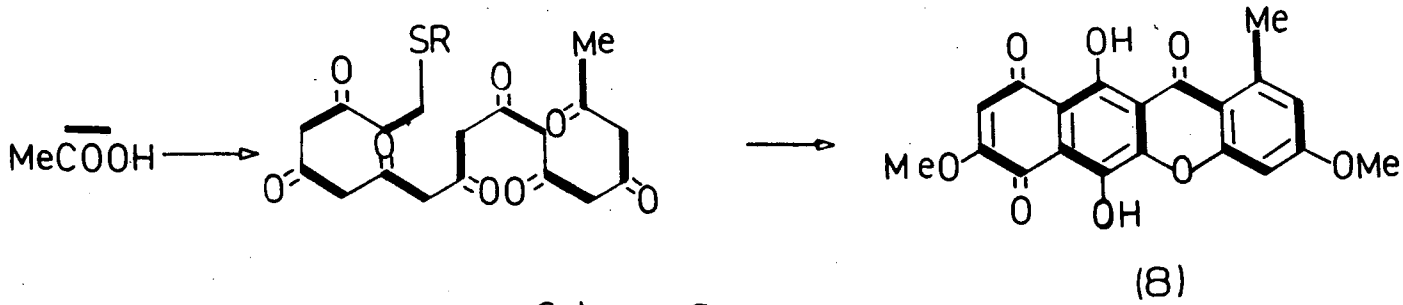
The xanthone or dibenzo-γ-pyrone ring system(1) is found as the basic structure of a number of natural products (secondary metabolites) which have been isolated from a wide variety of natural sources.[1] For example, jacareubin(2) which has been isolated from the heartwood Calophyllum brasiliense[2] and magniferin(3) from Magnifera indica L. (Anacardiaceae)[3,4] are two xanthones biosynthesised by higher plants. More examples can be found in a recent review.[5] Fungi are another common source of xanthones. Pinselin(4) from Aspergillus armarus[6] and ravenelin(5) from Helminthosporium ravenelii,[7] are both typical fungal xanthones, although the former has also been recently isolated from cell tissue cultures of the plant Cassia occidentalis.[8] Lichexanthone(6) which has been isolated from a number of lichens[9,10] is a typical example from lichens. The xanthones, which are found in various parts of plants or in the mycelia of moulds, are generally obtained by solvent extraction (Soxhlet method) of the dried and disintegrated material or are extracted from an aqueous substrate on which the fungi has been grown. A good example of the latter method is the isolation of pinselin(4).[6] The crude extract may often be purified by recrystallisation but a prior purification by chromatography is usually necessary. Since most naturally occurring xanthones sublime readily this method of purification can be used to purify some of them. All naturally occurring xanthones bear a hydroxy group in the 1-(or equivalent 8-position). They thus have certain properties in common with 1-hydroxy xanthone itself, e.g. they are often yellow in colour, the majority of them give a green colour with ferric chloride in ethanolic solution and they are coloured intensely yellow on



Scheme 1



Scheme 2



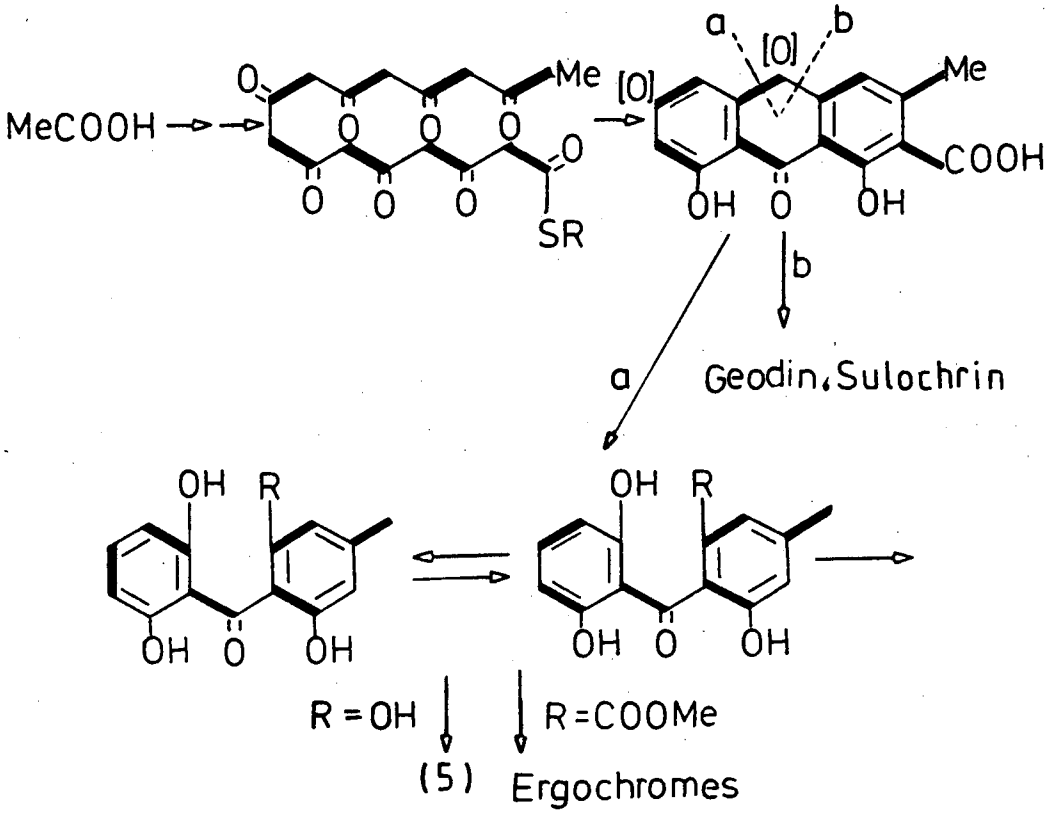
Scheme 3

contact with 2N sodium hydroxide solution.

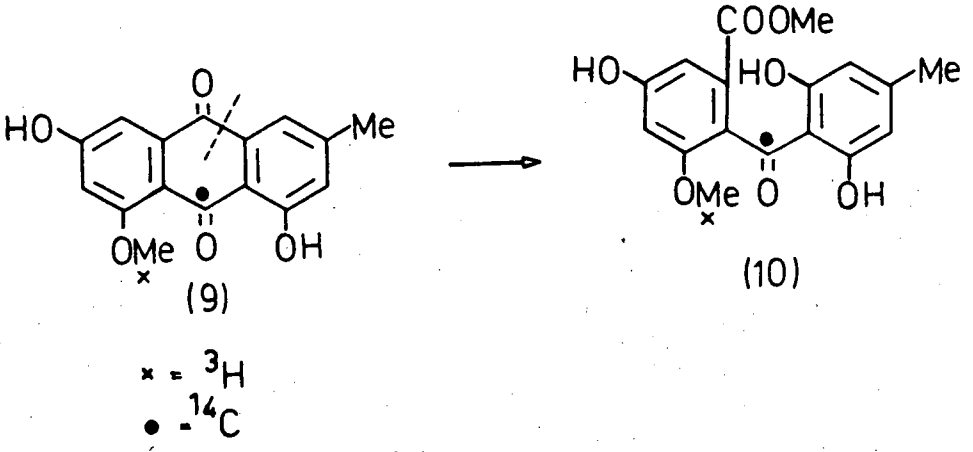
2.2 BIOSYNTHESIS OF XANTHONES

Hydroxylated benzophenones are generally accepted as the immediate precursors of most xanthones but their biogenesis in higher plants usually differs from that in fungi and lichens.[12] In higher plants benzophenones are derived by a mixed shikimate-polyketide pathway and the central xanthone ring is generated by oxidative coupling.[5,12] A necessity for this route is the presence of a hydroxyl group meta to the carbonyl function of the benzophenone and a second hydroxyl group on the other ring ortho to the carbonyl (Scheme 1). Jacarubin(2) and magniferin(3) are formed through this pathway.[13] This mixed biosynthesis can usually be recognised as they normally have 13 carbon atoms in the skeleton and by the different and characteristic hydroxylation pattern of the two benzene rings:- Ring B possesses oxygen atoms in alternative positions consistent with a polyacetate origin, whilst the ring A of shikimate origin has them in adjacent positions.

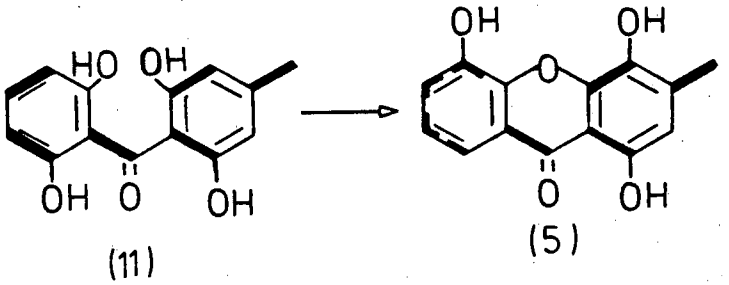
In microorganisms benzophenones are often formed by the polyketide pathway. The substitution pattern of these totally acetate-derived benzophenones presumably encourages ring closure by an addition-elimination process[11,14] as shown in Scheme 2 for lichexanthone(6). As is apparent in Scheme 2, the two hydroxyl groups in rings A and B of the intermediate benzophenone(7) are required to be ortho to the carbonyl function. This requirement is met in a looped folding of the fungal polyketide chain analogous to that observed[14] with bikaverin(8) Scheme 3. Xanthones formed via this route usually have an even number of carbon atoms in their skeleton and both rings A and B possess oxygen atoms in characteristic alternate positions. Another major route to xanthones involves



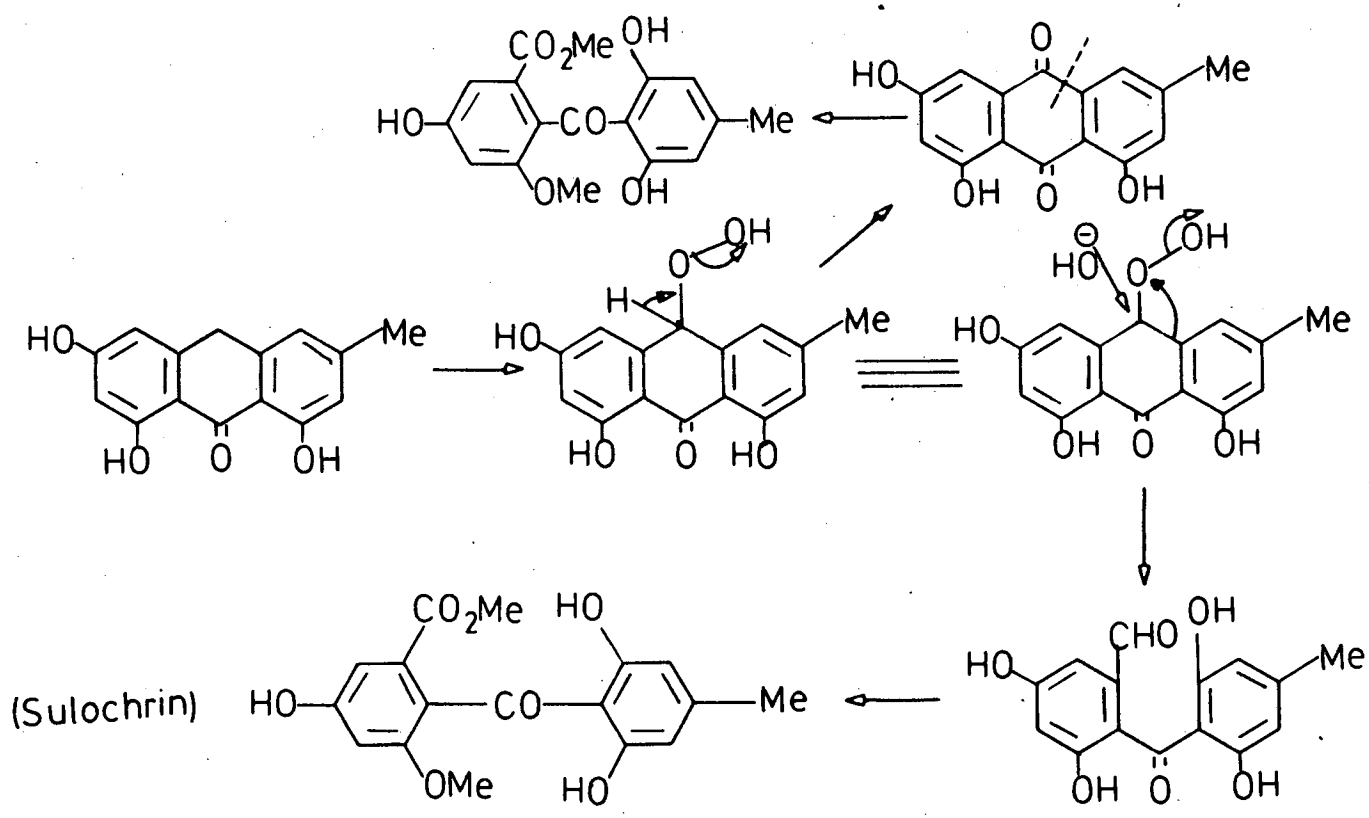
Scheme 4



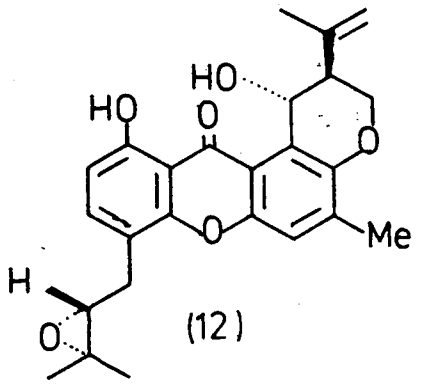
Scheme 5



Scheme 6


CO2Me
OH
HO
CO
Me
OMe
OH
O
HO
Me
OH
O
OH
HO
Me
O
OH
H
HO
O
HO
HO
Me
HO
O
OH
HO
O
OH
HO
Me
OH
O
OH
CO2Me
HO
(Sulochrin)
HO
CO
Me
OMe
HO
CHO
OH
HO
Me
OH
O
OH

Scheme 7



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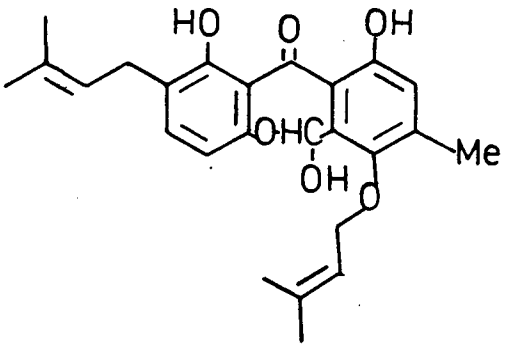
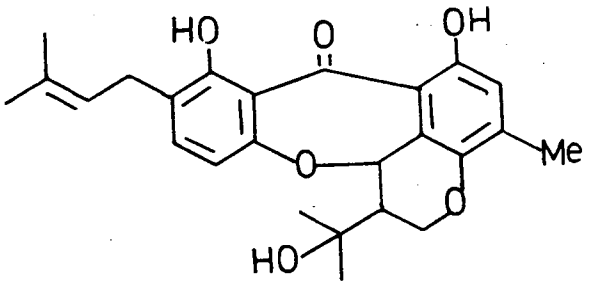


(13)



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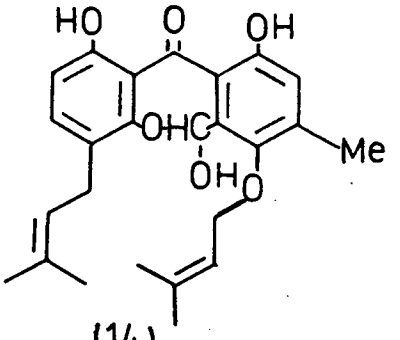

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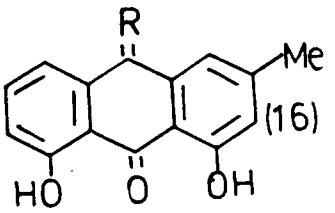
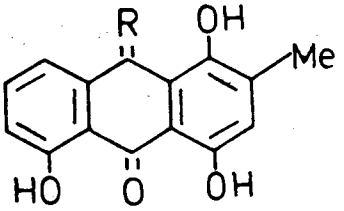


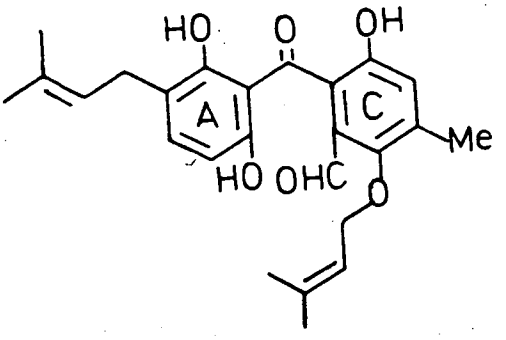
(16) R = O

(17) R = H_2



(18) R = O

(19) R = H_2

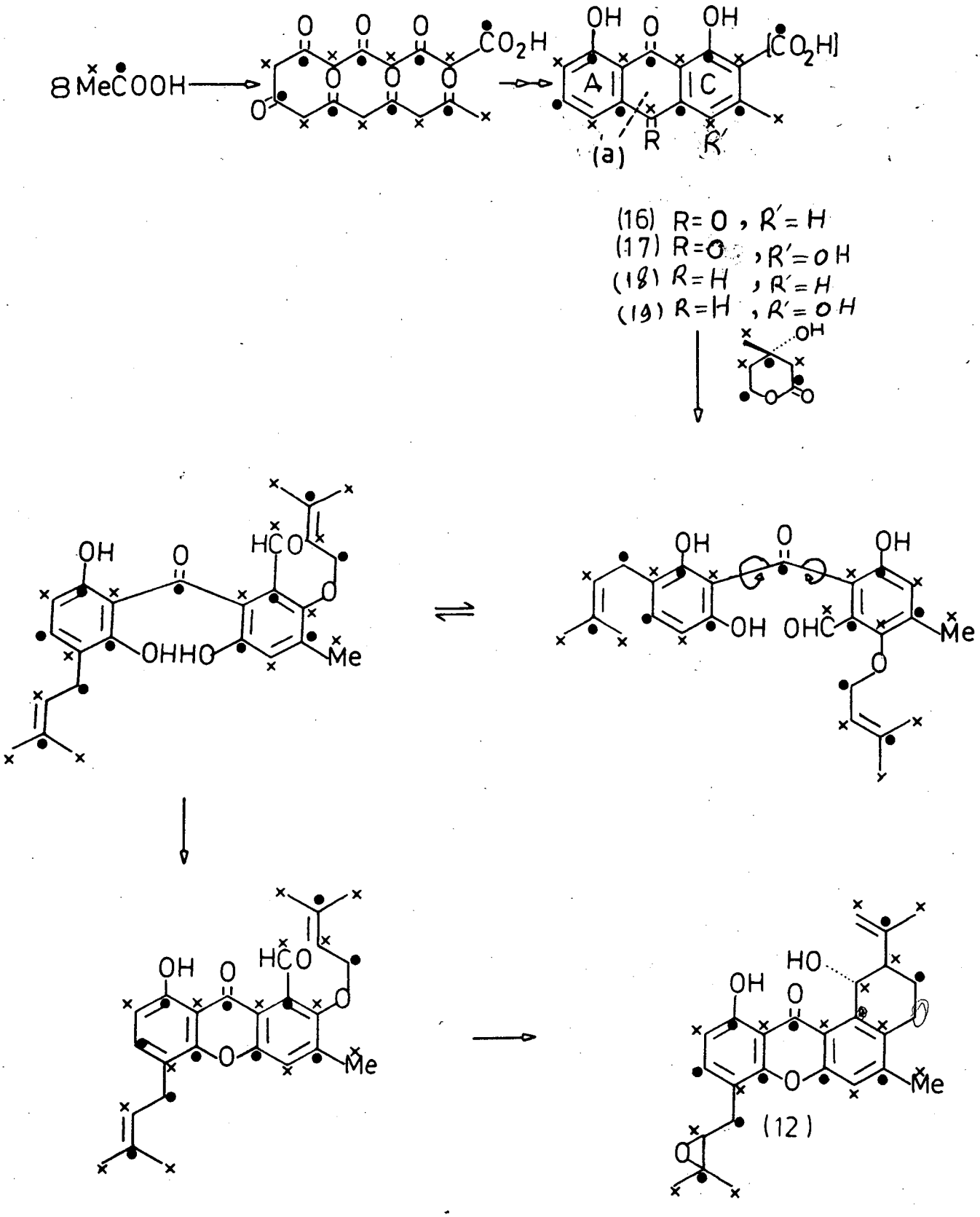


(20)

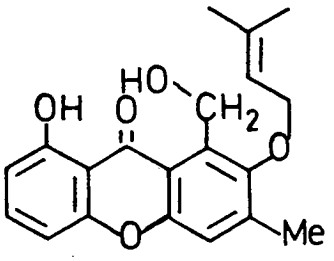
oxidative cleavage of an intermediate polyketide derived anthrone or anthraquinone.[15,16] After fission of the central ring by a still unknown mechanism, the resulting benzophenones often possess an axis of symmetry in one labile ring, rotation along this axis can put the two hydroxyl groups on both rings in the ortho position to the carbonyl function of the intermediate benzophenone, and hence will fulfil the requirement for a cyclodehydration to occur, Scheme 4. In fact this route has been demonstrated via a [^{14}C] labelling experiment involving the incorporation of questin(9) into sulochrin(10) Scheme 5.[17] The observed labelling pattern in ravenelin enriched with [1,2-$^{13}C_2$]acetate also indicates that an oxygenated benzophenone derivative(11) of polyketide origin, is an intermediate in the biogenesis of ravenelin(5), Scheme 6.[15] Possible mechanisms for the cleavage of the carbocyclic intermediate include biological Baeyer-Villiger oxidation[18] of the anthraquinones or cleavage via hydroperoxide adducts[19,20] of anthraquinone or anthrone intermediates, Scheme 7.

2.3 TAJIXANTHONE: INTRODUCTION

Tajixanthone(12) is one of a number of highly modified xanthones first isolated from cultures of Aspergillus variecolor.[21] Isolation of this compound and some of its co-metabolites from A.nidulans has also been reported.[22] Detailed analysis of ^{1}H and ^{13}C n.m.r. spectra of this metabolite and its derived compounds as well as chemical degradation shows the presence of a substituted dihydropyran system.[23] Further studies revealed that A.variecolor produced a number of structurally related minor metabolites,[24] together with known compounds arugosin A(13) and B(14).[25] The structures of this group of compounds, together with arugosin C(15)[26] suggests that they constitute a biogenetically related group. The xanthone system in


8 MeCOOH
CO2H
OH
A
C
R
R'
(a)
(16) R=O , R'=H
(17) R=O , R'=OH
(18) R=H , R'=H
(19) R=H , R'=OH
OH
HC
Me
HO
(12)

Scheme 8



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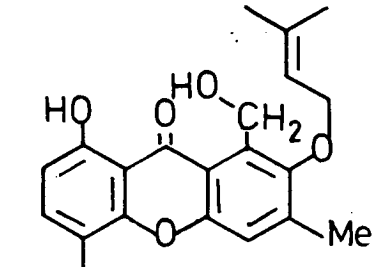

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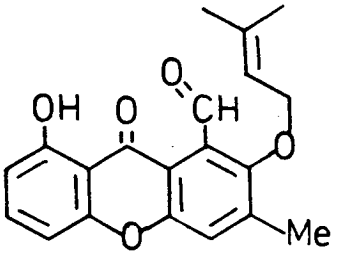

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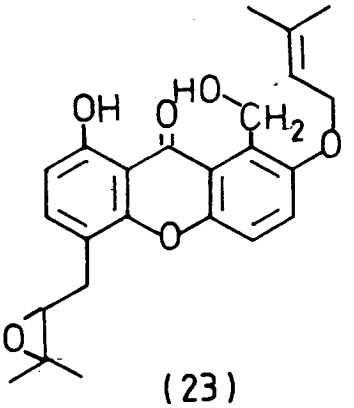

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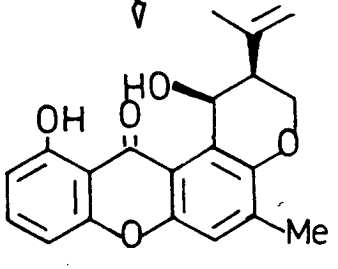


(25)

Scheme 9

tajixanthone could be formed by cyclodehydration of the O,O-dihydroxy-benzophenone system present as a hemiacetal in arugosins A(13) and B(14). The dihydropyran ring being formed by cyclisation of the O-prenyloxyaldehyde equivalent (20) present in arugosins A and B. Hence the possible polyketide origin of tajixanthone via an anthraquinone or anthrone arose from the thought that arugosins might be derived from an anthraquinone, for example chrysophanol(16) or islandicin(17) or the corresponding anthrones, (18) and (19) by oxidative ring cleavage with the introduction of two branched C-5 units from mevalonate via dimethylallyl pyrophosphate as shown in Scheme 8.

Incorporation studies[16] were therefore carried out using [1-^{13}C]- and [2-^{13}C]acetates and the analysis of the resultant ^{13}C n.m.r. spectra of enriched tajixanthone gave the labelling pattern which is shown in Scheme 8 and is in full agreement with the proposed biosynthetic pathway.

In addition, three other compounds were isolated from A.variecolor: variecoxanthones A, B and C with structures (21), (22) and (23) respectively[27] although the isolation of these xanthones is consistent with the idea that xanthone ring closure precedes dihydropyran ring formation, it was also shown that oxidation of variecoxanthone A(21) by Jones reagent gave the corresponding aldehyde(24) and the latter under mild acidic conditions isomerised to (25) in which the isopropenyl and hydroxyl substituents in the dihydropyran ring are cis related (Scheme 9). That fact that this compound is the cis product whereas tajixanthone is the trans product suggests that the dihydropyran ring formation may precede xanthone formation. In the transition state required for trans stereochemistry

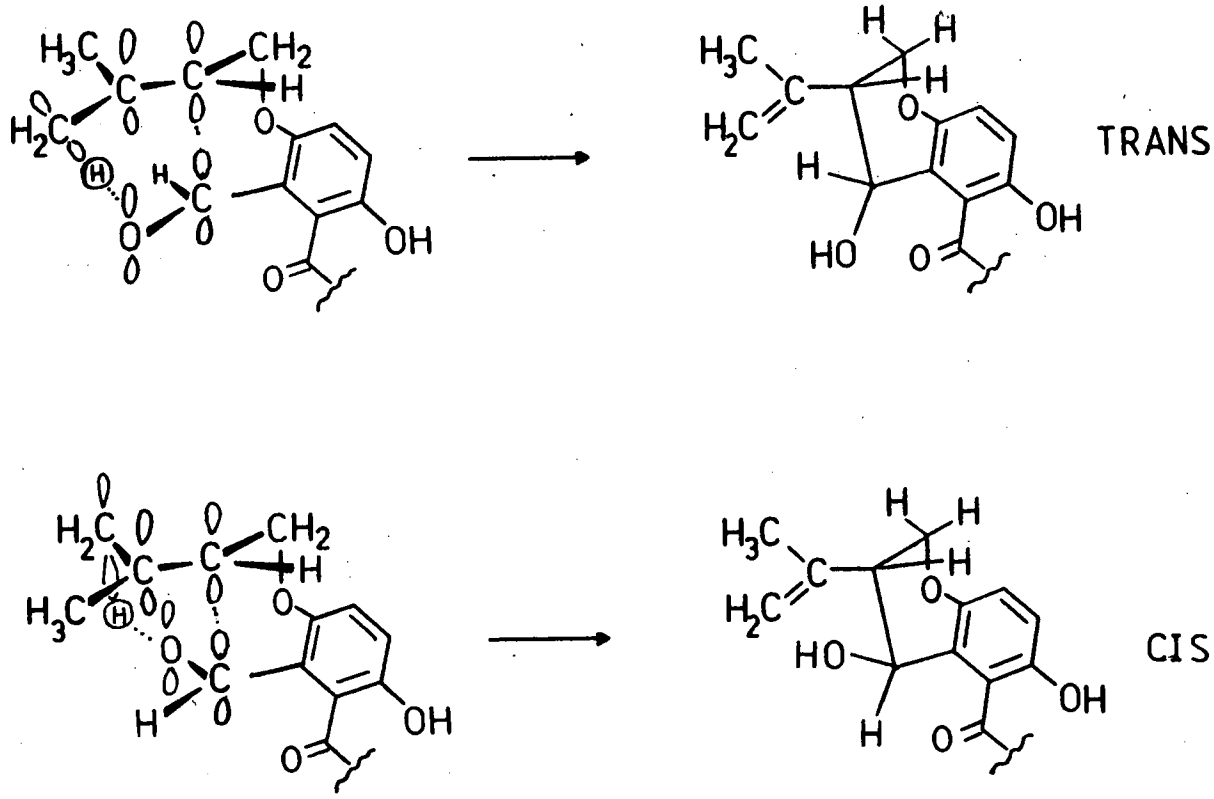
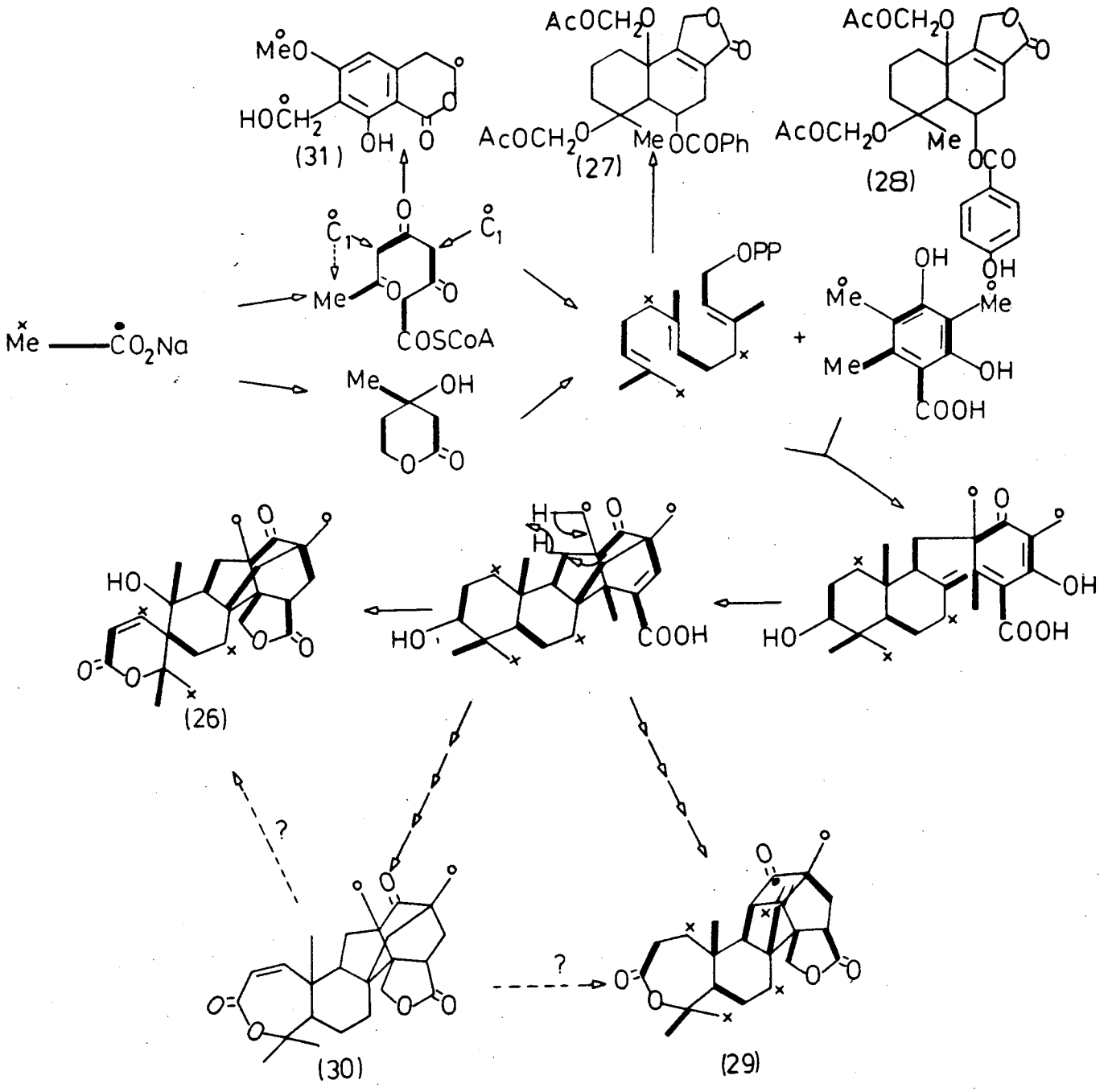


Figure 1 Transition state goemetries leading to cis and trans products in "ene" reactions of O-prenyloxyaldehydes.


MeO
HOCH2
OH
(31)
AcOCH2O
AcOCH2O
Me
OCOPh
(27)
AcOCH2O
AcOCH2O
Me
OCO
OH
(28)
Me
CO2Na
C1
C1
Me
COSCoA
OPP
OH
Me
Me
Me
OH
COOH
Me
OH
HO
(26)
HO
COOH
HO
OH
COOH
?
?
(30)
(29)

Scheme 10

in a concerted "ene" reaction[28] figure 1, there is a highly unfavourable interaction between the aldehyde and xanthone carbonyl group. Whereas the transition state for cis stereochemistry does not have this unfavourable interaction. However, if dihydropyran ring formation precedes xanthone formation then the carbonyl group of the precursor benzophenone can rotate away from the aldehyde and so permit the transition state necessary for trans stereochemistry. Thus the sequence and mechanistic details of the steps in tajixanthone biosynthesis remained to be determined.

2.4 ^{13}C AND ^{2}H LABELLING STUDIES

In order to throw light on this proposed pathway we set out to use [1,2-$^{13}C_2$]acetate and [2-$^{2}H_3$]acetate in a series of feeding studies with A.variecolor. Two major biosynthetic pathways are evident in A.variecolor, one leading to tajixanthone and related mycelial pigments, and the other to andibenin(26) and related compounds isolated from the fermentation liquors.[29] Screening studies of different strains of A.variecolor prior to feeding experiments revealed that:

a) The sesquiterpenoids astellolide A(27) and astellolide B(28) are present in the fermentation liquors of a mutant strain of A.variecolor which does not produce andibenin and is apparently impaired in polyketide biosynthesis. The structures were deduced from spectroscopic studies and were confirmed by a single crystal x-ray study.[30]

b) Anditomin (29) has been isolated[31] from a wildtype strain A.variecolor along with the previously isolated andilesin C(30).[29] The structure followed from ^{1}H and ^{13}C n.m.r. comparisons with known metabolites, ^{1}H spin-spin decoupling studies, and ^{13}C - ^{13}C couplings observed

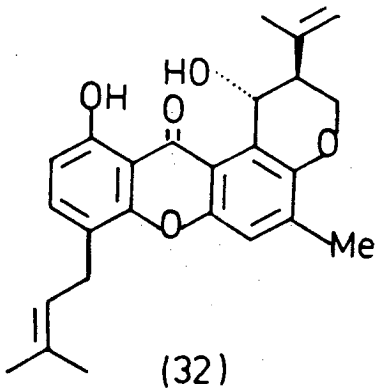
TABLE 1 Production of metabolites in static cultures of *A.variecolor*(212K)

Day[a]	Mycelium[b]	Tajixanthone (12)[c]	Shamixanthone (32)[c]	Arugosins (13)+(14)[c]	Sesterterpene[c]
2	3.1	0	0	0	0
4	4.8	19	1	14	2
7	8.3	25	10	27	8
9	12.2	59	12	33	7
11	12.1	68	32	14	3
14	13.2	65	12	7	5
16	11.9	37	2	1	1
18	12.3	64	1	1	1

a Innoculated on Day 0

b Yield in grams from 1 penicillin flask

c Yield in milligrams from 1 penicillin flask



(32)

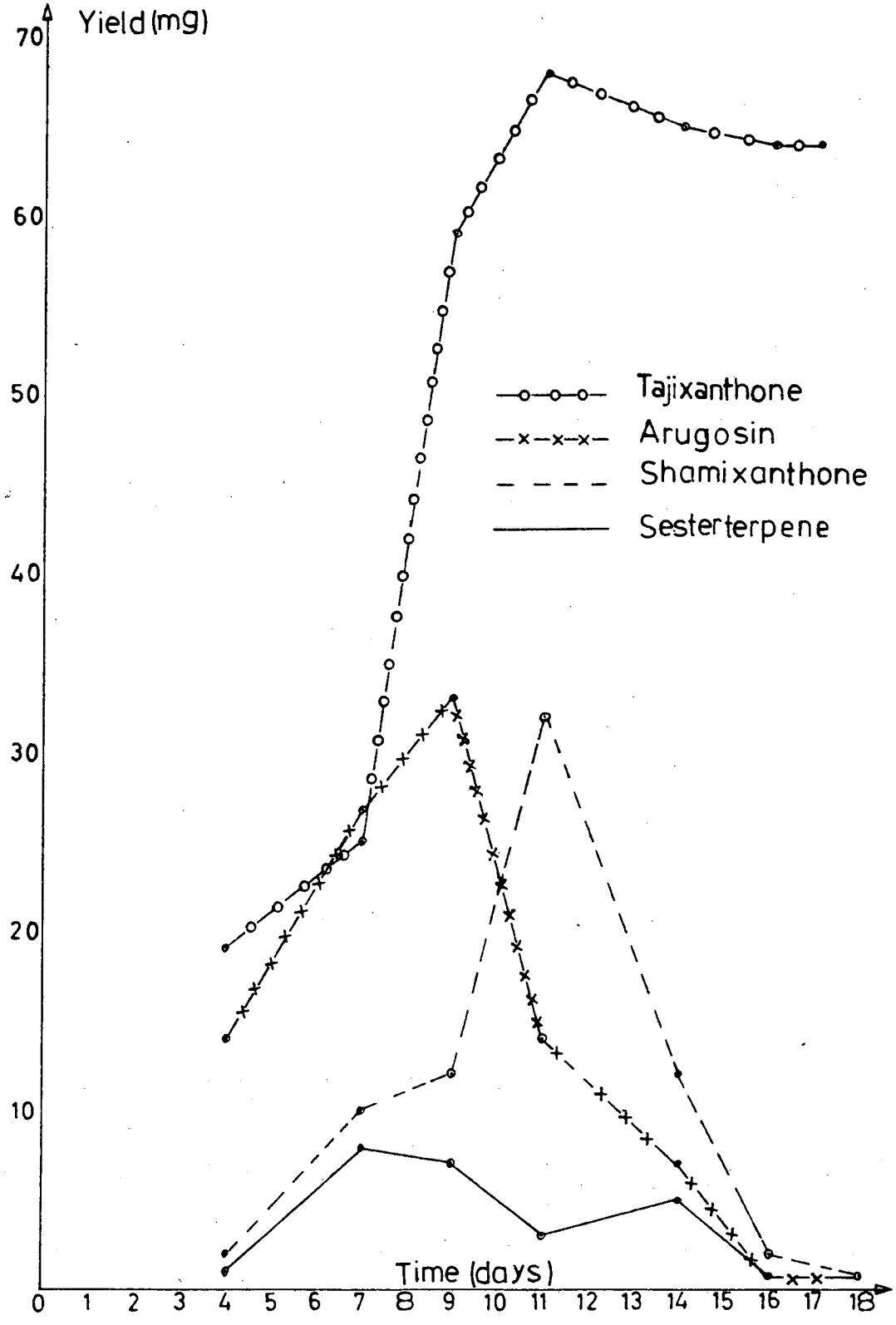


Figure 2 Time course of metabolite production in A variecolor 212K.

in [1,2-$^{13}C_2$]acetate enriched anditomin. The structure, which was confirmed by x-ray chrystallography represents an interesting variation of the andibenin biosynthetic pathway.[32]

c) Stellatin(31) has also been isolated from further wild-type strain of A.variecolor.[33] The unusual substituted dihydroisocoumarin structure was established mainly by analysis of the fully ^{1}H coupled ^{13}C n.m.r. spectrum.

The presumed biosynthetic relationship among the varied group of compounds is summarised in Scheme 10.

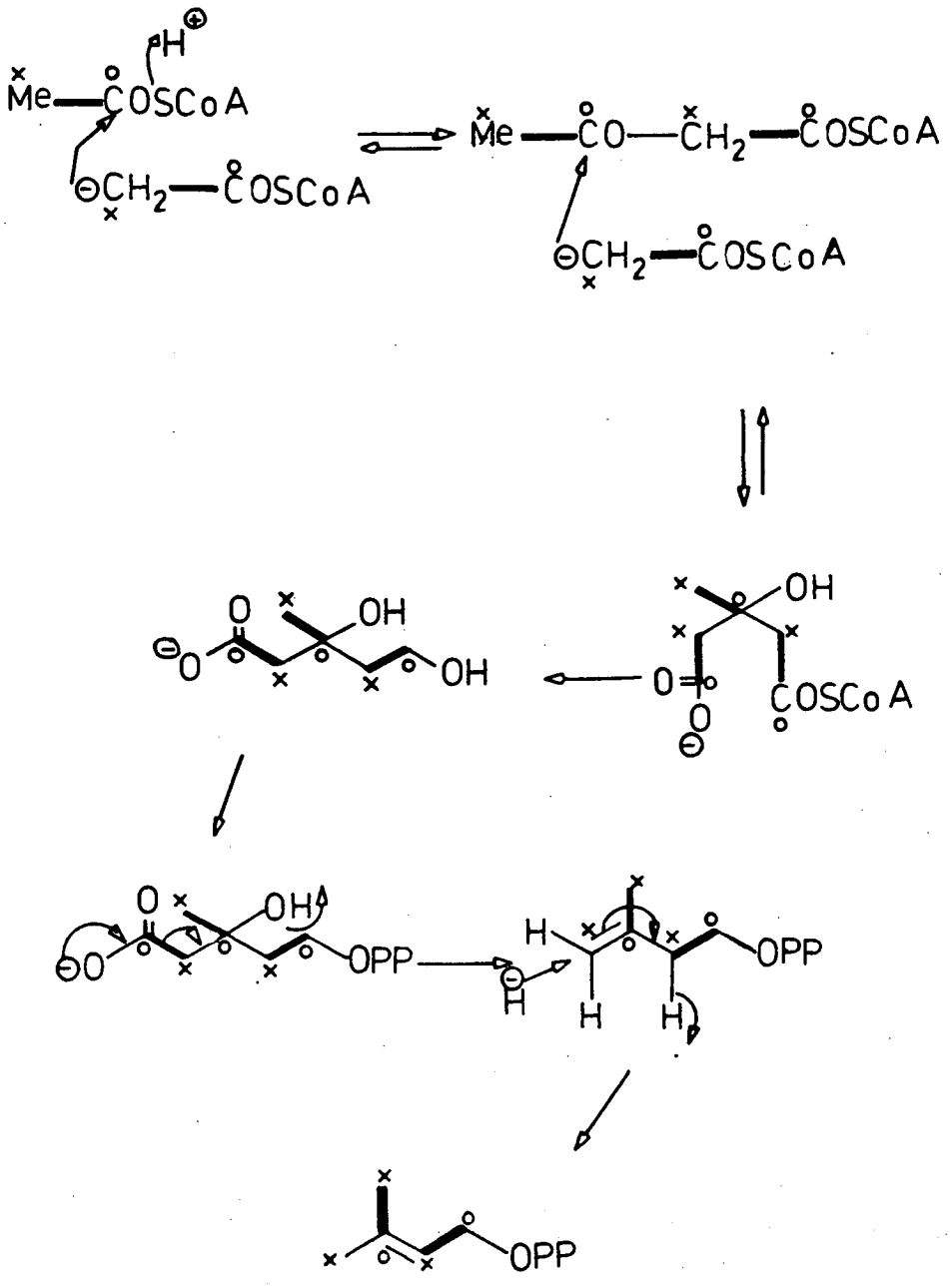
Soxhlet extraction of the dried and ground mycelium of A.variecolor (212K) with light petroleum and subsequent purification of the extract by thin layer chromatography showed that in general four metabolites were present - tajixanthone(12), shamixanthone(32), arugosin A and B, (13) and (14), and another metabolite C_{25} $H_{40}O$ which appears to be a sesterterpene, but its structure has not been fully established.[34] (Note that tajixanthone is simply the epoxy derivative of shamixanthone).

In order to determine the exact time that the production of tajixanthone and its co-metabolites starts, the mycelium was harvested at 2, or 3 days intervals, and extracted. Fractionation of the extracts and separation of the above mentioned metabolites gave the results depicted in Table 1 and shown in figure 2.

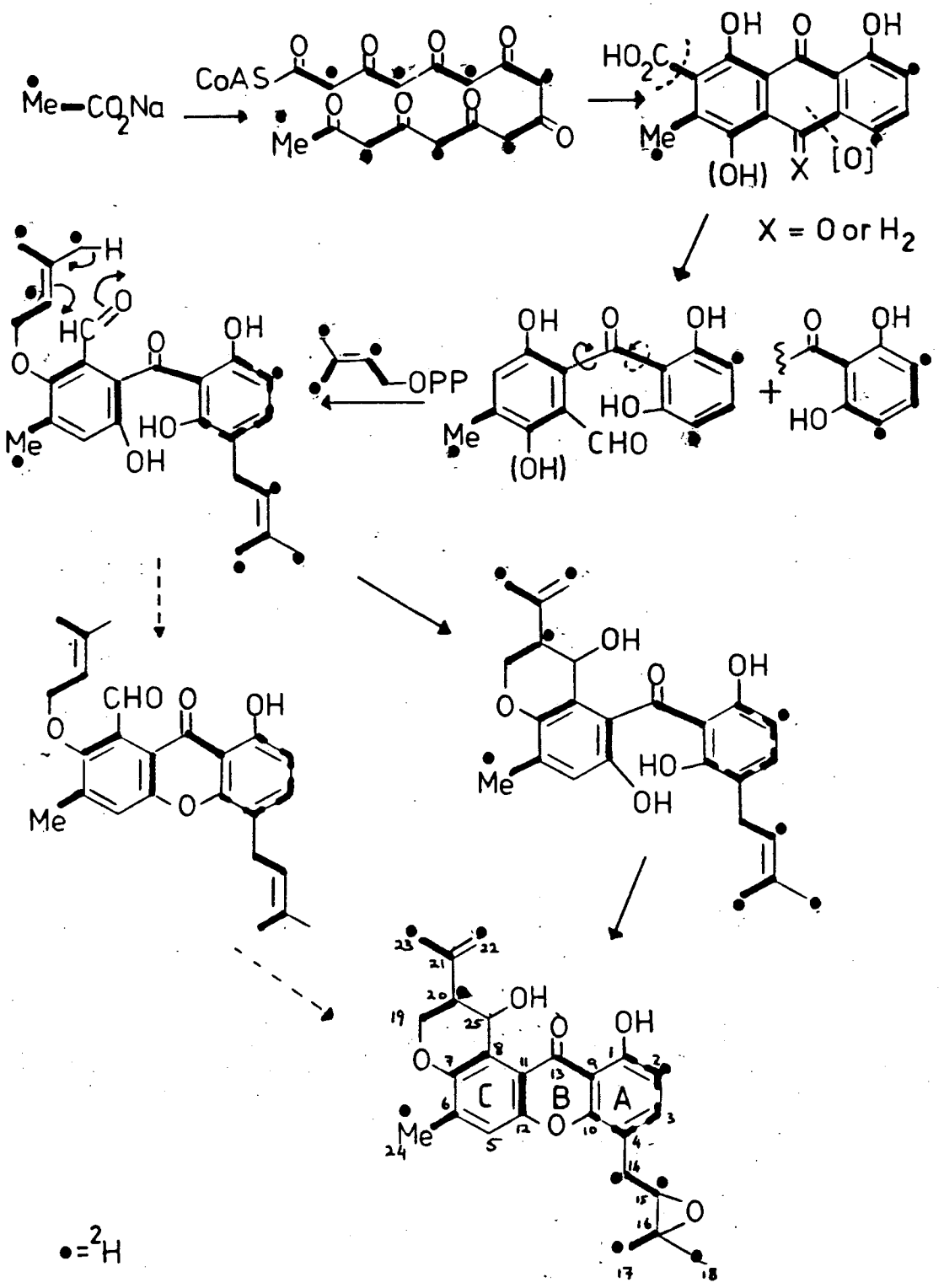
The growth production curve, figure 2 clearly shows that shamixanthone is produced after arugosins A and B and that as the level of tajixanthone in the mycelium of A.variecolor increases the amount of arugosins A and B, and shamixanthone decreases, to provide further support for the previous proposals that arugosin A is an intermediate during the biosynthesis of tajixanthone. The decrease in the level of shamixanthone can also be taken as evidence that

TABLE 2 ^{13}C chemical shifts and ^{13}C- ^{13}C couplings observed in the 90 MHz ^{13}C n.m.r. spectrum of [1,2-$^{13}C_2$]acetate enriched tajixanthone.

Carbon	Sc(p.p.m.)	J(Hz)
1	159.7	69.6
2	109.7	69.5, 57.4
3	136.4	60.8, 57.4
4	114.8	70.8, 60.8
5	118.7	-
6	137.8	43.9
7	149.0	68.5
8	120.6	68.5
9	108.7	57.3
10	152.3	70.8
11	151.4	64.7
12	116.4	64.5
13	183.6	57.3
14	28.5	43.9
15	63.0	43.9
16	58.5	45.2
17	19.0	45.2
18	24.7	-
19	64.3	34.2
20	44.8	34.2
21	142.0	42.7
22	111.8	-
23	22.4	42.5
24	17.3	43.9
25	63.0	-



Scheme 11


Me—CO2Na
CoAS
Me
HO2C
OH
O
OH
Me
(OH)
X
[O]
X = O or H2
H
HC
O
OH
HO
Me
OH
OPP
OH
O
OH
HO
Me
CHO
(OH)
O
OH
HO
CHO
O
OH
Me
O
OH
O
OH
HO
Me
OH
23
22
21
20
OH
19
25
O
OH
O
7
8
11
13
9
1
2
C
B
A
6
12
10
3
Me
24
5
4
14
15
O
16
17
18
•=2H

Scheme 12

epoxidation of this compound (which has been formed first) yields tajixanthone.

As the growth production curve shows the production of tajixanthone commences on day 4 but a sharp increase in the level of tajixanthone occurs on day 7. Preliminary experiments using [1-^{14}C]acetate showed that the maximum incorporation of the precursor occurs when the radioactive precursor is fed on day 6 of the fermentation. Further experiments, varying the amount of labelled acetate, revealed that doubly ^{13}C- labelled acetate would require to be fed at a level of 1g/litre to give sufficient enrichment for ^{13}C-^{13}C coupling satellites to be observed.[35] Accordingly [1,2-$^{13}C_2$]- and [2-$^{2}H_3$]acetates were fed to cultures of A.variecolor and the enriched tajixanthone was isolated in each case and the ^{13}C n.m.r. and ^{2}H n.m.r. spectra determined. The couplings observed in the ^{13}C n.m.r. spectrum of [1,2-$^{13}C_2$]acetate enriched tajixanthone are summarised in Table 2 and Scheme 12. ^{13}C-^{13}C couplings are observed between carbons 6 and 24, 7 and 8, 11 and 12, and 9 and 13 indicating that they are derived from intact acetate units. The ^{13}C-^{13}C coupling satellites of the ring A carbons show that carbons 1 and 2, 2 and 3, 3 and 4, 4 and 10 are all coupled but that the intesities of the satellites are only half as high as those of the four units in rings B and C, i.e. the couplings in ring A are randomised. ii) Carbons 19 and 20, 21 and 23, in the O-prenyl residue and carbons 14 and 15, 16 and 17 in the C-prenyl residue are coupled. This is consistent with the formation of these two units from mevalonic acid via dimethylallyl pyrophosphate, as shown in Scheme 11.

The 55 MHz ^{2}H n.m.r. spectrum of [2-$^{2}H_3$]acetate enriched tajixanthone is shown in figure 3a, compared with the 60 MHz ^{1}H n.m.r. spectrum (figure 3b). Figure 4 shows the spectrum redetermined in

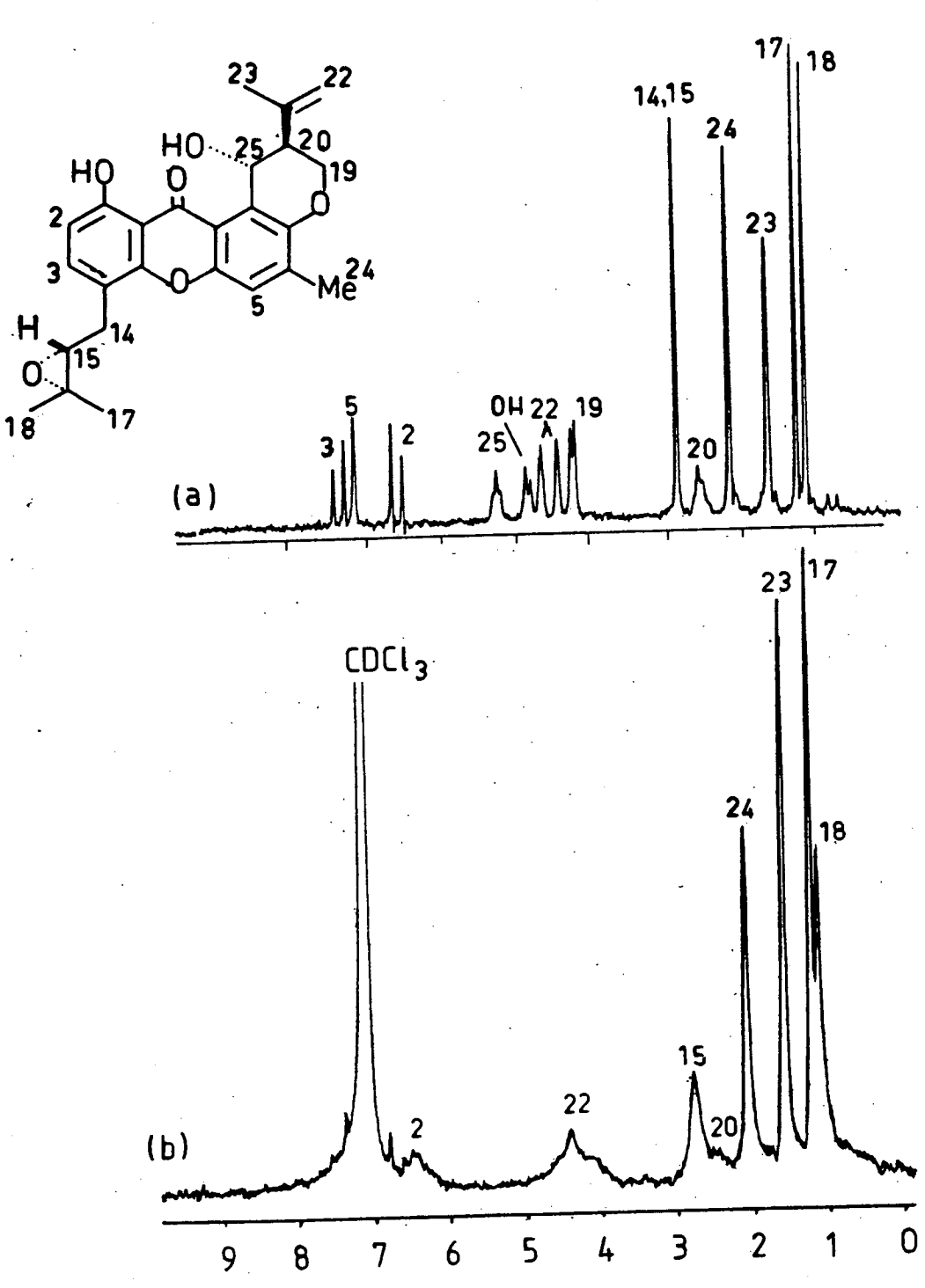


Figure 3 (a) 60MHz ^{1}H n.m.r. spectrum of tajixanthone (12) in $CDCl_3$, (b) 55 MHz ^{2}H n.m.r. spectrum of [$^{2}H_3$]acetate-enriched tajixanthone (12) in $CHCl_3$.

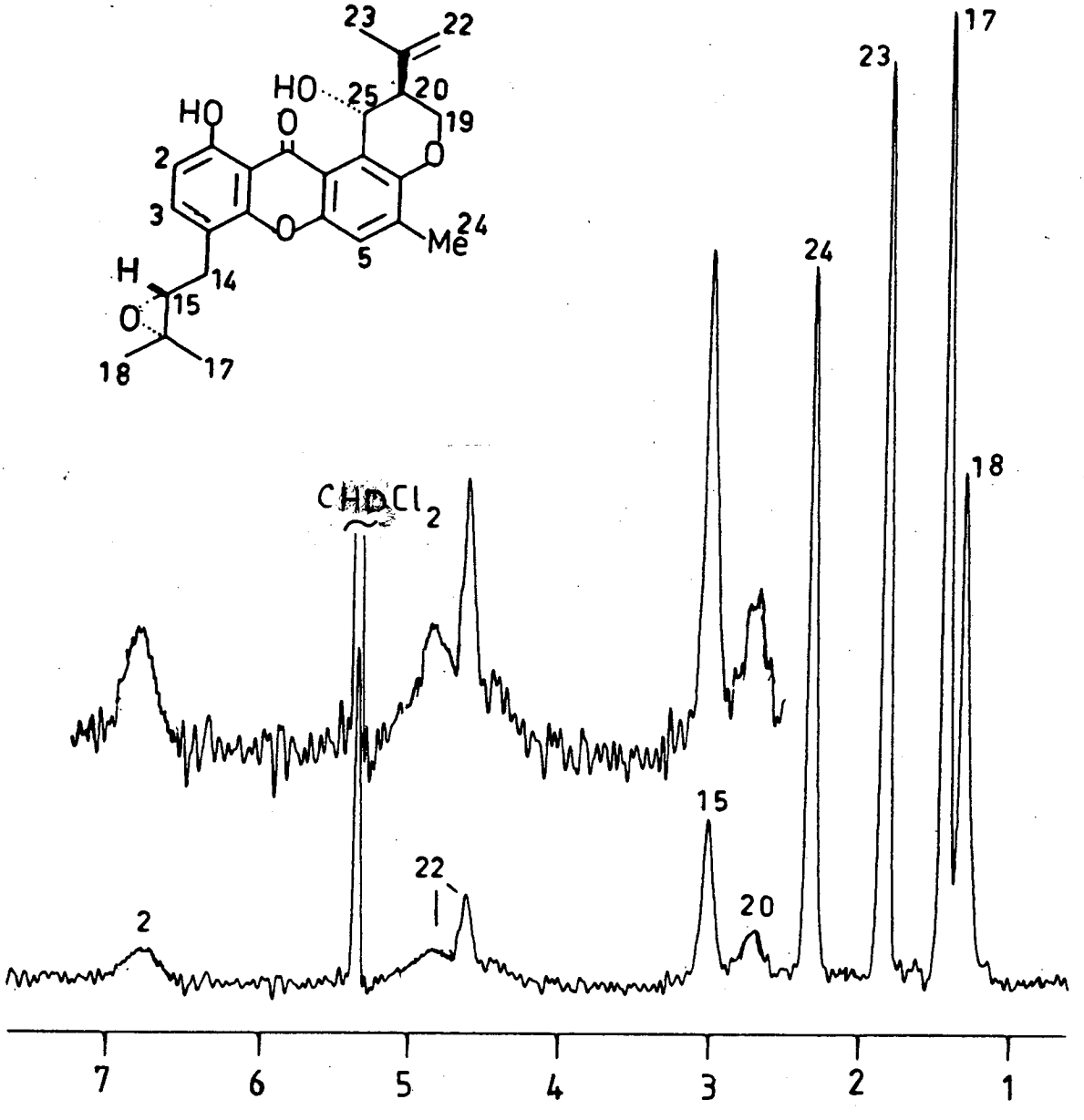
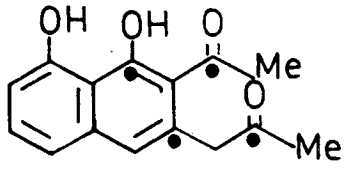
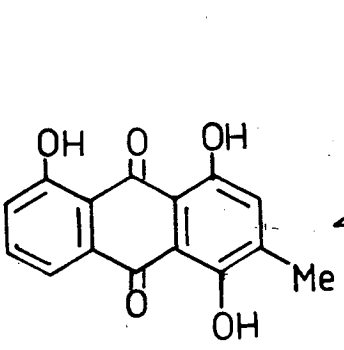


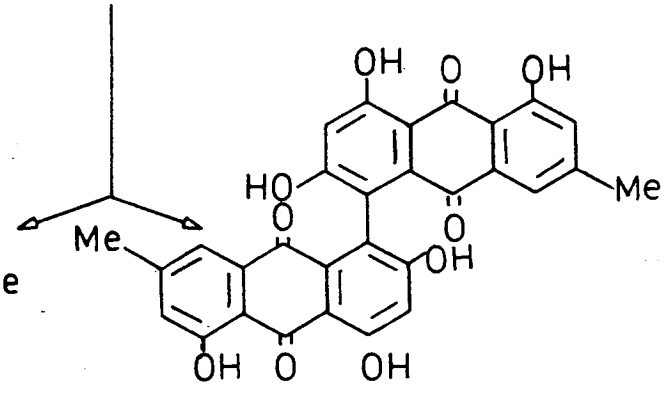
Figure 4 55 MHz ^{2}H n.m.r. spectrum of [$^{2}H_3$]acetate enriched tajixanthone (12) in CH_2Cl_2



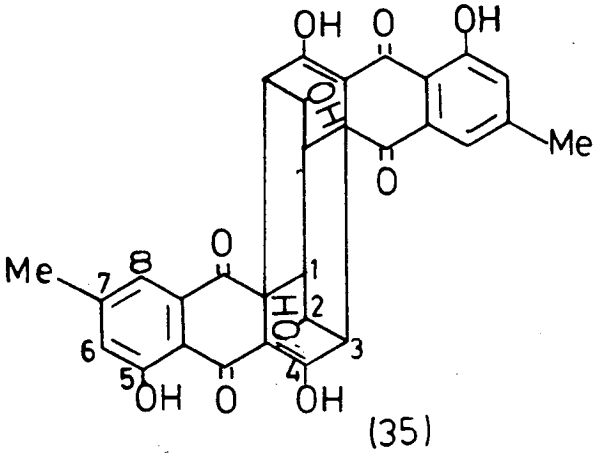
(33)



(18)



(34)



(35)

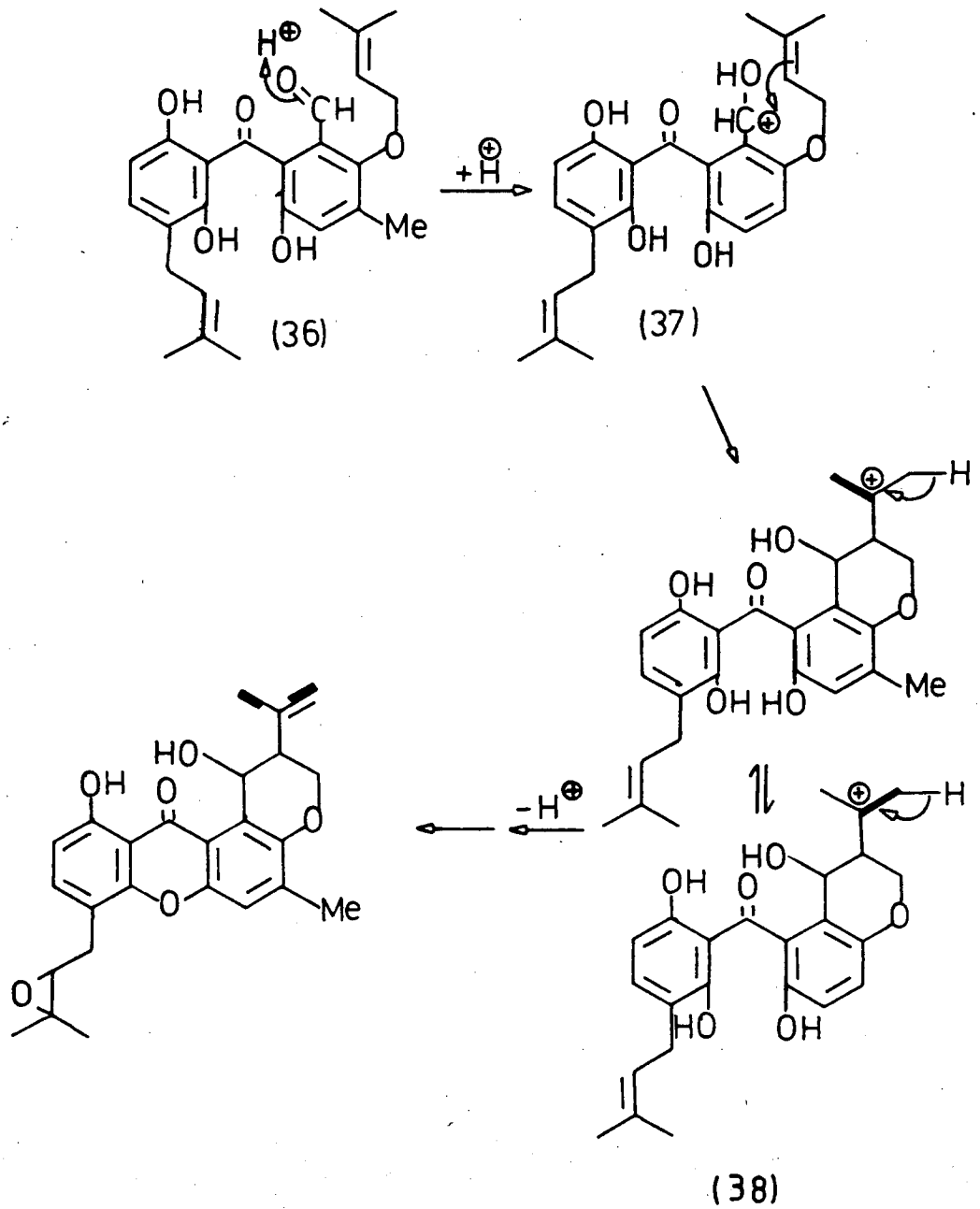
CH_2Cl_2 to show if anything was obscured by the solvent ($CHCl_3$) signal. These spectra indicate:-

a) There is no ^2H on C-25 (from aldehyde intermediate) or on C-5 (this is the carbon to which the chain terminating carboxyl was attached).

b) The extent of ^2H incorporation at different positions is not equal, i.e. ^2H has been incorporated at C-2, C-15, C-17, C-20, C-22, C-23 and C-24 and to a lesser extent, at C-18.

The following conclusions can be drawn from the above:-

i) The acetate assembly pattern in the xanthone system is entirely consistent with an octaketide precursor folded as shown in Scheme 12. Decarboxylation of the octaketide precursor occurs after cyclisation and aromatization, since redetermining the ^2H n.m.r. spectrum of tajixanthone in CH_2Cl_2 shows that there is no ^2H on C-5, the carbon to which the terminal carboxyl of the octaketide precursor was attached. This contrasts with results reported by Franck on anthraquinone biosynthesis in Penicillium islandicum.[36] ^{14}C-Labelled diketonaphthol(33) was fed to surface cultures to yield radioactive islandicin(18) and skyrin(34). However, no degradations to confirm the specificity of incorporation were reported. On the other hand when [2-^{13}C, $^2H_3$]acetate was incorporated into rugulosin(35) by cultures of Penicillium brunneum,[37] deuterium was present at C-1, C-3, C-8 and the 7-methyl but not at C-6 indicating again that the necessary decarboxylation of the octaketide precursor occurs after formation of the aromatic rings.



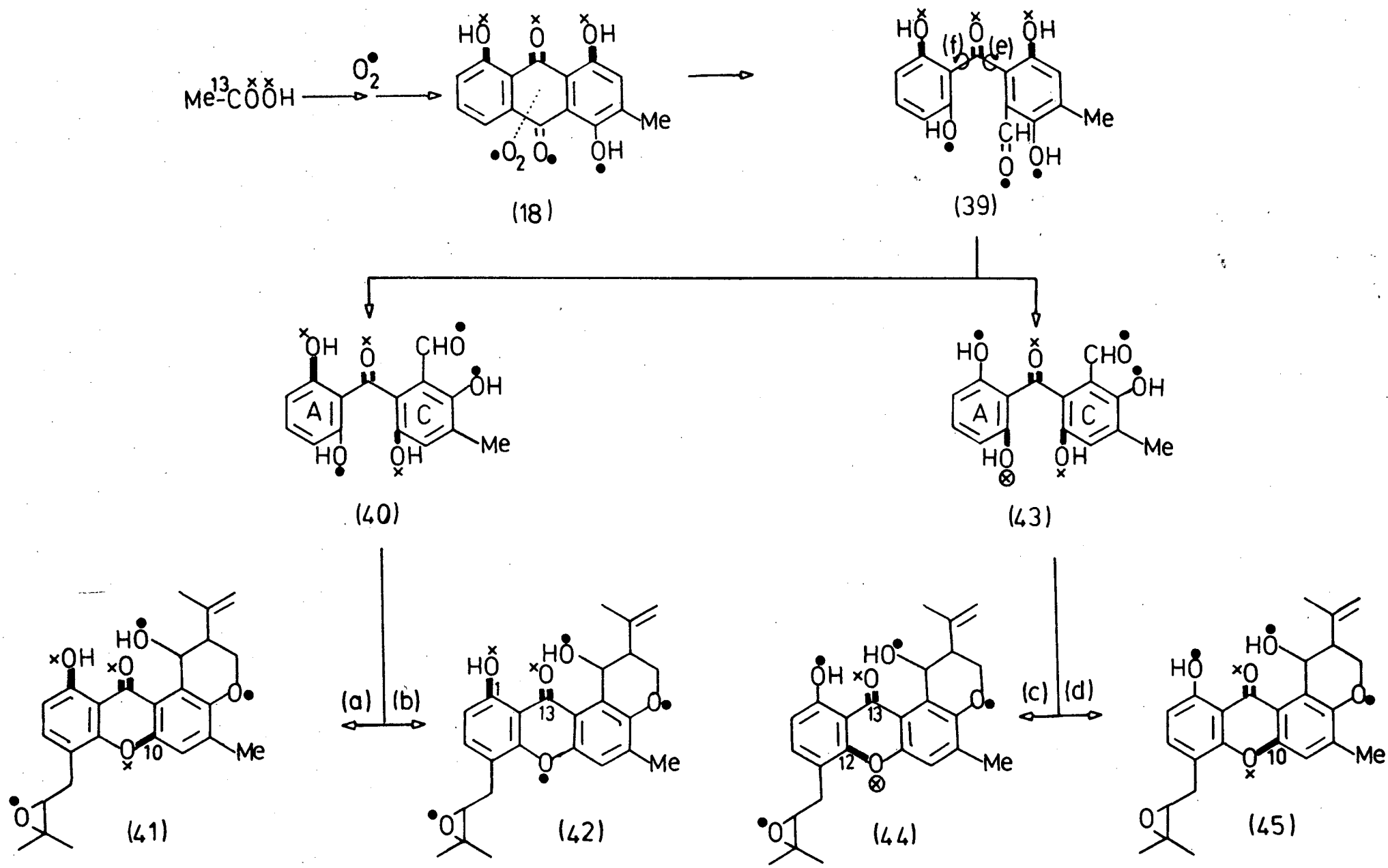
Scheme 13

ii) No deuterium is present at C-25. This suggests that the oxidative cleavage occurs on an anthraquinone rather than on an anthrone intermediate.

iii) The randomisation of labelling in ring C means that ring C must have been symmetrical and free to rotate on the enzyme surface at some stage in the biosynthesis of tajixanthone. Therefore ring cleavage of the carbocyclic precursor must precede the introduction of the C-prenyl residue.

iv) C-prenylation and epoxidation occurs with retention of configuration about the double bond of dimethylallyl pyrophosphate in full agreement with Scheme 12.

v) The stereospecificity of ^{13}C - labelling in the dihydropyran ring suggests its formation from the O-prenyl aldehyde moiety by a concerted "ene" reaction. Discussion of the mechanism of the formation of the same ring via a stepwise reaction will help to clarify this proposal. In an acid catalysed addition of the double bond to the carbonyl (Scheme 13) protonation of the aldehyde oxygen of (36) forms a carbonium ion (37) which will be attacked by the electron rich double bond to give (38). Now in the newly formed carbonium ion (38) the methyl groups (one of them part of an intact acetate unit) become equivalent so each of them can participate equally in the last step of the reaction which is the loss of a proton. If this were the mechanism of the reaction for the formation of the dihydropyran ring, the single bond ^{13}C-^{13}C coupling between C-23 and C-21 (derived from an intact acetate unit) would have been randomised, whereas ^{13}C n.m.r. shows that C-23 and C-21 are still



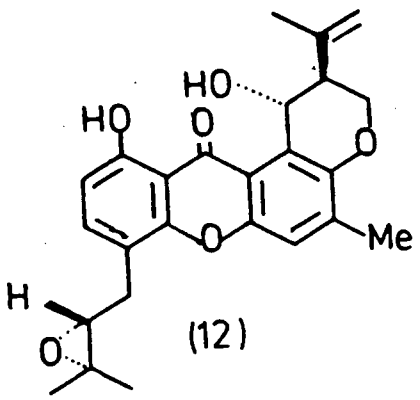
Scheme 14

coupled but there is no evidence for any coupling between C-21 and C-22.

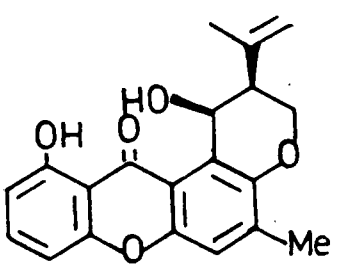
As discussed above, to obtain the correct stereochemistry in a concerted reaction requires dihydropyran ring formation before cyclodehydration to the xanthone system, as the transition state necessary for trans stereochemistry in an "ene" reaction of the xanthone aldehyde would have a highly unfavourable interaction between the aldehyde and xanthone carbonyls. Indeed attempted cyclisation of model compounds give equal amounts of the cis and trans stereochemistry, this will be discussed in the following section.

2.5 ^{18}O LABELLING STUDIES

Further mechanistic insights could be obtained by feeding [1-^{13}C, $^{18}O_2$]acetate to A. variecolor and checking the ^{13}C n.m.r. of the hopefully enriched tajixanthone to determine the origin of the oxygen atoms (for the details of the methodology see the introduction). After oxidative ring cleavage of the carbocyclic precursor and therefore the introduction of two oxygen atoms which are not labelled, two different pathways could be followed (Scheme 14). Oxidative cleavage of an anthraquinone precursor, e.g. islandicin(18) would give the benzophenone intermediate (39) which on rotation about axis(e) is converted to (40) as shown in Scheme 14. Xanthone ring formation can then occur by either (a) nucleophilic attack of the ring C hydroxyl on ring A or (b) of the ring A hydroxyl on ring C. If path (a) operates then the xanthone ring oxygen will carry ^{18}O label and C-10 will show an ^{18}O isotope in the ^{13}C n.m.r. spectrum of the enriched tajixanthone(41). However, if path (b) operates then the ^{18}O atom is lost and only C-1 and C-13 will show isotope shifts in the enriched tajixanthone(42). Alternatively as shown in Scheme 14 rotation could occur about both axis (e) and axis (f) in the initially



(12)



(25)

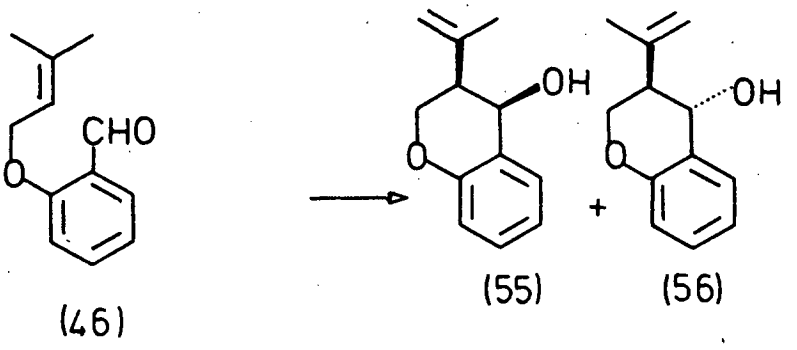
formed benzophenone(39) to give (43) as shown. Again nucleophilic attack of the ring A hydroxyl on ring C, path (c); or of the ring C hydroxyl on ring A, path (d) could occur. Path (c) would result in isotope shifts being observed for C-12, in addition to C-13 in the enriched tajixanthone(44). Path (d) would result in isotope shifts being observed for C-10, and C-13 in the enriched tajixanthone(45). So since the precursor acetate is doubly labelled with ^{13}C and ^{18}O, and the chance of incorporation of two acetates in the same molecule is small, the two modes of xanthone ring closure may be distinguished by the observation of an ^{18}O isotope shift on either C-10 or C-12.

In an effort to maximise the incorporation of acetate to a level suitable to permit observation of ^{18}O induced shifts, the incorporation was studied under a number of conditions. However, none gave very high incorporation. [1-^{13}C, $^{18}O_2$]acetate was incorporated but on analysis of the enriched tajixanthone, only C-13 showed an ^{18}O isotope induced shift (0.027 p.p.m.) and even this was of very low intensity. It is assumed that the very low incorporation and the possible small size of the ^{18}O induced shifts[38] precluded observation of the crucial shifts on C-1, C-10 and C-12.

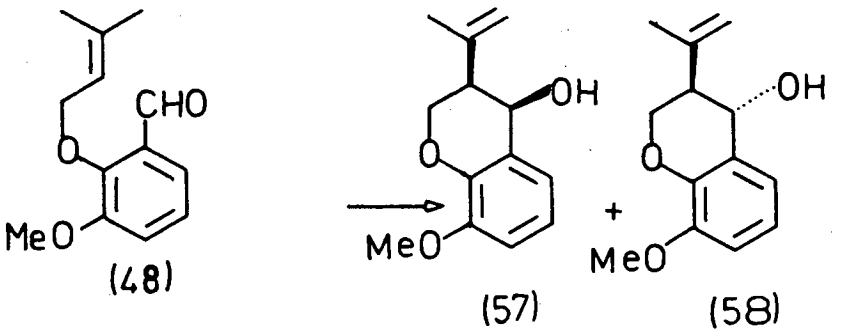
2.6 BIOMIMETIC CYCLISATION EXPERIMENTS

The simulation of biosynthetic steps by model compounds (*in vitro*) is a very useful way for better understanding the mechanisms of the reactions that take place during biosynthetic processes.[39]

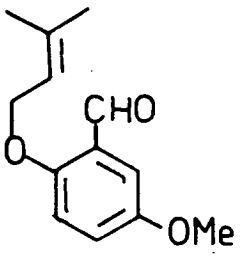
The predominant formation of only one isomer during the dihydropyran ring closure in tajixanthone (*trans* isomer) and the variecoxanthone derivative (24) (*cis* isomer) suggest that the dihydropyran ring in both of them is formed through a cyclic intermediate in a concerted reaction. So in order to produce more evidence for this suggestion a series of model compounds were



(46) (55) (56)

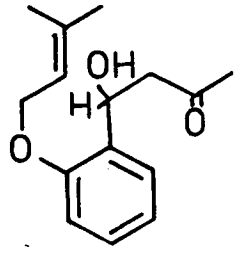


(48) (57) (58)

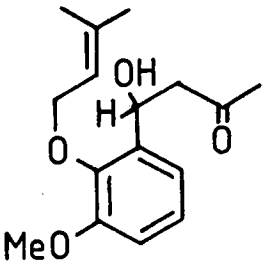


(47) → No Reaction

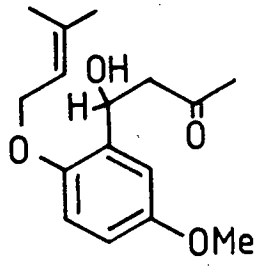
Scheme 15



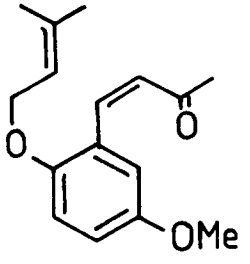
(49)



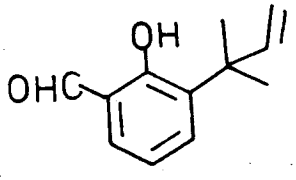
(50)



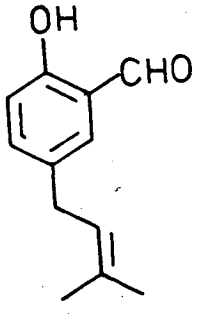
(51)



(52)



(53)



(54)

synthesised and the ring closure process was examined *in vitro*. Thus three model compounds, *O*-prenyloxysalicylaldehyde(46), 5-methoxy-*O*-prenyloxysalicaldehyde(47) and 3-methoxy-*O*-prenyloxy salicylaldehyde(48) were prepared by alkylation of the corresponding phenols with 1-bromo-3-methylbut-2-ene.

During these condensation reactions a number of highly crystalline by products were also formed. It was shown that an increase in the amount of potassium carbonate would increase the percentage of these by products. Spectroscopic evidence allowed structures (49)-(52) to be assigned to these compounds. It was obvious that all these compounds were the result of an aldol condensation between acetone and the aldehydes. The formation of these by products was minimised by using only one equivalent of potassium carbonate.

Attempted cyclisation of compound (46) using dilute hydrochloric acid in chloroform only resulted in the corresponding Claisen rearrangement products (53) and (54). Reaction occurred to both ortho and para positions although the ortho product predominates. However, it was found that paratoluene sulfonic acid was quite effective for bringing about cyclisation.[40] The reaction was carried out at room temperature by stirring each of the *O*-prenyl aldehydes (46), (47) and (48), Scheme 15, with a catalytic amount of p-toluene sulfonic acid in dry benzene. Compound (47) did not undergo cyclisation but compound (46) gave two cyclisation products (55) and (56) in a one to one ratio, but in very low yields (less than 2%). Although the formation of the dihydropyran ring in compound (48) was slow, the yield of the two predicted *cis* and *trans* isomers (57) and (58) (in 1:1 ratio) was quite high (70% mixture of the two isomers). The higher yield of this reaction is probably due to the presence of the bulky

methoxy group in the ortho position of the O-prenyl residue because the former pushes the latter up towards the aldehyde group, i.e. brings them close enough towards each other for the cyclisation reaction to occur. The fact that the formation of the dihydropyran ring in compound (24) occurs, readily under mild conditions (chloroform, R.T.), whereas in the aldehyde(48) it is much slower, could be explained by the fact that the aldehyde carbonyl is conjugated with the aromatic ring in (24) but is not conjugated in (48). This is shown clearly by the comparison between the infra red spectrum of the variecoxanthones with that of the model compounds which shows that in the former the carbonyl group of the aldehyde is not conjugated (1710 cm^{-1}) with the aromatic ring whereas in the latter it is (1690 cm^{-1}). The formation of the dihydropyran ring is confirmed not only by the disappearance of the aldehyde signal in the ^{1}H n.m.r. (10.48) p.p.m., but also comparing the infra red spectra of the starting aldehydes with the two compounds (57) and (58) shows the absence of the carbonyl group, (1690 cm^{-1}) in (57) and (58). The infra red spectrum of (57) shows a sharp hydroxyl stretching band at 3520 cm^{-1}, characteristic of free or non hydrogen bonded hydroxyl groups, while the hydroxyl stretching band for compound (58) appears as a broad absorption at3350 cm^{-1}, characteristic of associated or hydrogen bonded hydroxy groups. The presence of hydrogen bonding in compound (58) and lack of it in (57) could be explained by the fact that the hydroxyl group in (57) is in a crowded environment (cis configuration) which prevents hydrogen bonding, but this hindrance is not present in (58) which allows the molecules to get close enough for the formation of hydrogen bonds (trans configuration). This is further confirmed by comparing the melting points. Compound (57) (presumed cis isomer)- has a melting point of 63-65^{o}C and (58) (trans

TABLE 3 ^{1}H n.m.r. assignment for *cis* isomer (57).

Hydrogens	H(p.p.m.)	No. of protons	Multiplicity (J in Hz)
OH	1.89	1	d (2.9), exchangeable
Me	1.90	3	dd (1.2, 0.7)
Hb	2.69	1	dt (11.5, 3.0)
OMe	3.87	3	s
Hc	4.28	1	dd (11.5, 10.5)
Hd	4.36	1	ddd (10.4, 3.0, 1.3)
He	4.73	1	Multiplet
Ha	4.76	1	ddd (3.0, 2.9, 1.3)
Hf	5.07	1	Multiplet
H-6	6.82	1	dd (7.9, 1.8)
H-5	6.89	1	t (7.9)
H-4	6.92	1	dd (7.9, 1.8)

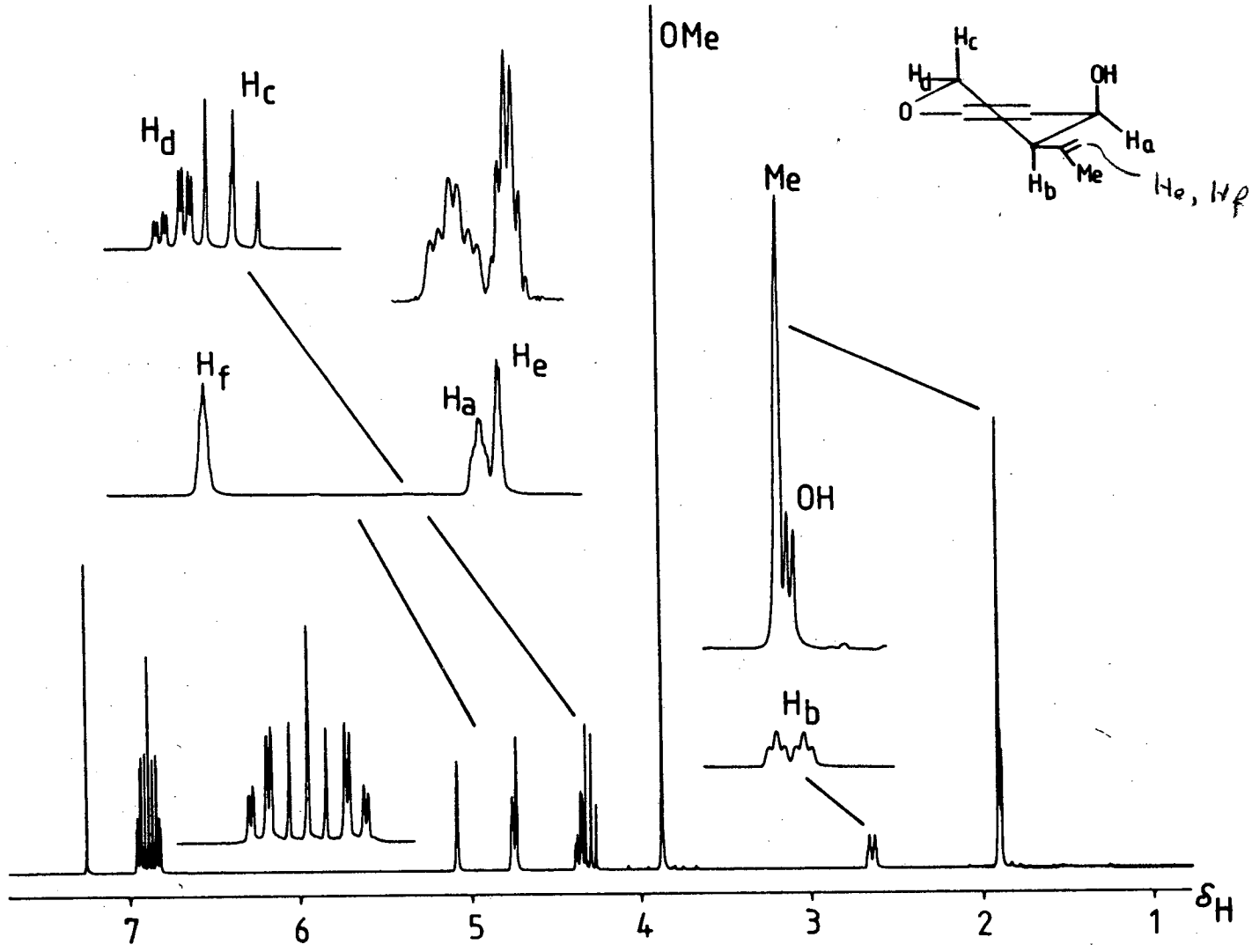


Figure 5 360 MHz ^{1}H n.m.r. spectrum of cis isomer (57).

TABLE 4 ^{1}H n.m.r. assignment for *trans* isomer (58).

Hydrogens	H(p.p.m.)	No. of protons	Multiplicity (J in Hz)
Me	1.85	3	dd (1.2, 1.0)
OH	2.13	1	s, exchangeable
Hb	2.58	1	td (8.4, 3.6)
OMe	3.80	3	s
Hc	4.14	1	dd (11.2, 8.4)
Hd	4.35	1	dd (11.2, 3.6)
Ha	4.81	1	d (8.4)
He	4.84	1	broad singlet
Hf	4.99	1	pentet (1.5)
H-6	6.79	1	dd (8.0, 1.7)
H-5	6.90	1	t (8.0)
H-4	7.05	1	dd (8.0, 1.7)

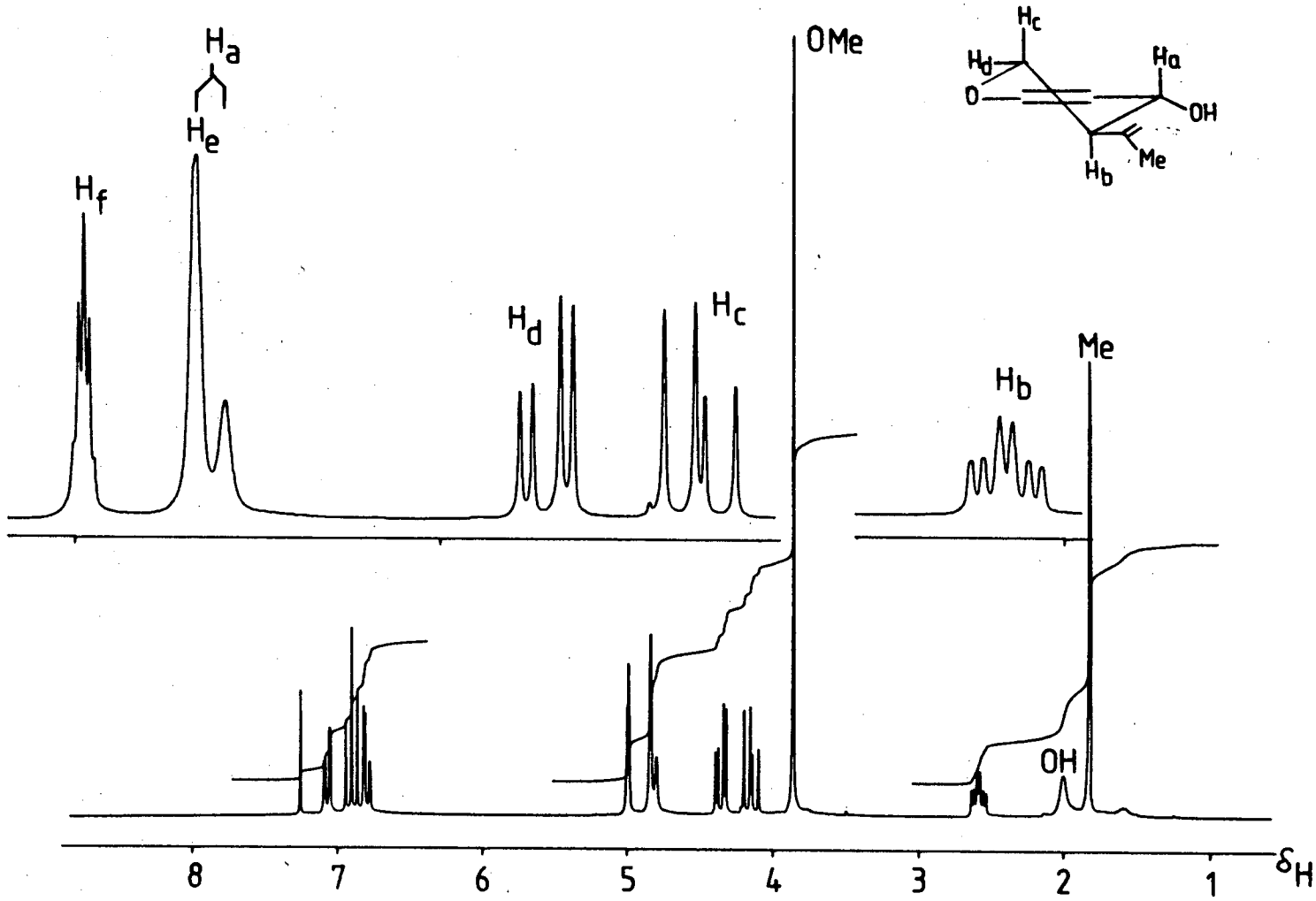
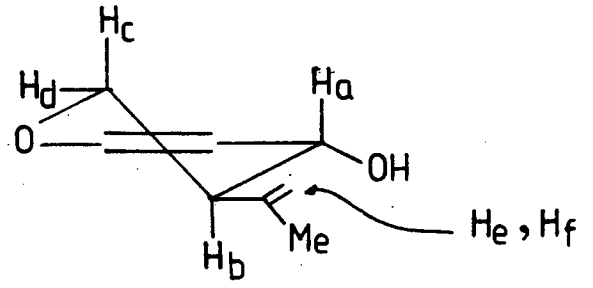


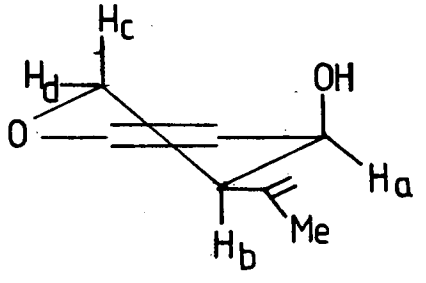
Figure 6 200MHz ^{1}H n.m.r. spectrum of trans isomer (58).

(a)



(58) TRANS

(b)



(57) CIS

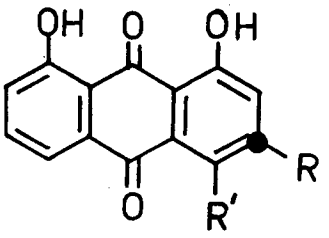
Figure 7 Relative stereochemistry of the isopropenyl and hydroxyl substituents in (57) and (58).

isomer) melts at 82-84°C in full agreement with more stable trans configuration.

These assumptions are further confirmed by direct comparison between the n.m.r. spectra of the two isomers. The ^1H n.m.r. spectra of (57) and (58) determined at 360 MHz and 200 MHz respectively are shown in Figures 5 and 6, and summarised in Tables 3 and 4. Compound (58) is shown to be the trans isomer with the hydroxyl and isopropene substituents occupying pseudo-equatorial positions as indicated in Figure 7a. H_b, attached to, the carbon bearing the isopropene substituent shows trans-diaxial coupling [41] to both H_a, attached to the hydroxyl bearing carbon; and to H_c. This is in contrast to tajixanthone itself where detailed n.m.r. studies have shown the substituents on the dihydropyran ring system to occupy pseudo-axial positions.[24] The couplings observed (Table 3) for (57) indicate that it is the cis isomer. H_b now shows a trans diaxial coupling to H_c, the axial hydrogen on the methylene carbon, so that the isopropene substituent must be pseudo-equatorial. However, H_b also shows a gauche coupling to H_a, so that the hydroxyl group must occupy a pseudo-axial position as depicted in Figure 7b. The assignment of a pseudo-equatorial position to H_a is further evidenced by the observation of a "W" coupling (1.3 Hz) between H_a and H_d the equatorial methylene hydrogen. In confirmation of the infra-red data, the hydroxyl proton must be in a hindered position and not readily exchangeable as it shows a coupling (2.9 Hz) to H_a. The substituents on the dihydropyran ring of the cis-product(25) derived from variecoxanthone A have also been shown to have the same disposition as in the cis isomer (57).[28]

2.7 ATTEMPTED INCORPORATION OF LABELLED ANTHRAQUINONES

As the incorporation experiments with [2-$^2H_3$]acetate described



(59) $R = CH_3$, $R' = H$, ● = ^{14}C

(60) $R = CD_3$, $R' = H$, ● = ^{12}C

(61) $R = CH_3$, $R' = OH$ ● = ^{14}C

(62) $R = CD_3$, $R' = OH$, ● = ^{12}C

above failed to produce evidence for the intermediacy of anthrones as the carbocyclic precursors for the ring cleavage step, a number of experiments using ^{14}C- and ^{2}H-labelled anthraquinones were also conducted. [3-^{14}C]-chrysophanol(59), [methyl-^{2}H]-chrysophanol(60), [3-^{14}C]-islandicin(61), [methyl-^{2}H]-islandicin(62), were all prepared as described in the following chapter. Our preliminary incorporation experiments proved negative, with no detectable incorporation of label into tajixanthone being observed. However, incorporation of [^{14}C]-chrysophanol into tajixanthone, shamixanthone, and arugosins A and B have been reported by Franck.[42] The negative results in our series of experiments could be due to:

i) These compounds are highly insoluble in water and only soluble (with difficulty) in organic solvents, such as acetone or methanol. Feeding the solution of substrates in these solvents can upset the organism.

ii) Permeability barriers within and between the cells can stop these rather large molecules penetrating cell walls.

iii) Even if the precursor penetrates to the synthetic site it may be that at that time the enzyme systems mediating the synthetic reactions are not present and active. In the light of the above experiments with ^{13}C - and ^{2}H - labelled acetates, it would appear that chrysophanol and/or islandicin are precursors to tajixanthone. More work is required to optimise the feeding conditions for labelled anthraquinones to see if we can reproduce Franck's results.

2.8 GENERAL EXPERIMENTAL PROCEDURES

Melting points were determined on a Reichert hot-stage microscope and are uncorrected. Microanalyses were performed on a

Perkin-Elmer 204 elemental analyser. Ultra-violet spectra were determined on a Varian DMS90 spectrophotometer. Infra-red spectra were measured on a Perkin-Elmer 781 spectrophotometer and referenced against the polystyrene absorption at 1601cm^{-1}. Proton n.m.r. spectra were obtained on Varian EM360 and HA100 continuous-wave machines and Bruker WP80SY, WP200SY, WM/WB 300 and WH360 Fourier-transform machines. Deuterium n.m.r. spectra were determined on a Bruker WH360, operating in this case, without a frequency rock. Carbon-13 n.m.r. spectra were obtained from Bruker WP200SY and WH360 machines. In all cases, quoted chemical shifts are relative to tetramethylsilane, δ_H and δ_C = 0.0 p.p.m. Mass spectra were determined on an A.E.I. MS902 high-resolution spectrometer. Radioactivity was measured using a Beckman LS7000 liquid scintillation counter. Counting efficiency was determined by the added external standard method using [^{14}C]hexadecane. The scintillant was butyl-PBD (10gl^{-1}) in methanol-toluene (50:50). Samples for radiocounting were purified to constant activity by recrystallisation and dissolved in methanol or toluene. Unless otherwise specified, thin layer chromatography (t.l.c.) was carried out using either analytical (5 x 20cm) or preparative (20 x20cm) glass plates coated with a 0.5mm layer of silica-gel (Merck Art. 7730 Kieselgel GF254 or Fluka AG 60765 Kieselgel GF254). Chromatograms were visualised by ultra-violet light on wavelength 254 n.m. or by spraying with ethanol-sulphuric acid (85:15) and heating on a hot plate. Solutions for feeding studies were sterilized by autoclaving at 15 p.s.i. for 15 minutes. Solvents were purified and dried by standard procedures.

2.9 EXPERIMENTAL

Isolation of Tajixanthone

Asperigillus variecolor, (strain 212K); was grown from a spore

suspension in static culture for 15 days at 25°C in flat vessels (Ca 1 l capacity) each containing Czapex-Dox medium (500ml). The dried mycelium (Ca 11-12g); was ground and continuously extracted with light petroleum (b.p. 40-60°). The resulting dark oil was triturated with warm methanol, and the methanol soluble fraction evaporated to give a yellow solid which was fractionated by preparative t.l.c. using benzene-ether (95:5;v.v) as developing solvent. Tajixanthone was eluted with chloroform from the band with RfO.4 and recrystallised from methanol to give yellow needles m.p. 157-159°C (lit.[24] m.p. 159°C).

Incorporation of [1-^{14}C]Acetic Acid Sodium Salt

Preliminary experiments on cultures of A.variecolor grown in static culture as previously described showed that tajixanthone production commenced on the fourth day after inoculation from a spore suspension and reached a maximum on the eleventh day. Furthermore feeding of [1-^{14}C]acetate showed that maximum incorporation into tajixanthone occurred when the label was introduced six days after inoculation, and the compound was isolated from 12-day cultures. When sodium[1-^{14}C]acetate (5g; 5.59μCi mmol^{-1}) was added to five culture vessels each containing a 6-day growth of the organism on Czapex-Dox medium (500ml; 5% sucrose) and the mycelium was harvested after a further six days, tajixanthone (Ca 30mg; 0.13μCi mmol^{-1}) was obtained. This corresponds to a dilution factor[36] for label of 43.

Incorporation of Sodium [2-$^{2}H_3$]Acetate

To each of 10 culture vessels containing a 6-day growth of A.variecolor(Strain 212K) was added sodium [2-$^{2}H_3$]acetate (0.5g). After a further six days the mycelium (56g) was harvested and the tajixanthone isolated (391mg after purification). This sample was used for deuterium n.m.r. studies.

Incorporation of Sodium [1,2-$^{13}C_2$]Acetate

Sodium [1,2-$^{13}C_2$]acetate (250mg) was added to a culture vessel containing a six day growth of A.variecolor (212K) and mycelium was harvested after a further six days and tajixanthone (86mg) was obtained as usual.

Incorporation of [3-^{14}C]Islandicin (61)

A solution of [3-^{14}C]islandicin (50mg; 7.7 x 10^6d min.$^{-1}$ min.mol^{-1}) in acetone (40ml) was prepared. Immediately prior to addition a 5ml aliquot of the above solution was mixed with an equal amount of detergent solution (2% in water) and this mixture was added to 10 flasks every 12 hours for four days starting on day four (1ml of mixture to each flask). The mycelium was harvested on day nine, dried, and extracted to give tajixanthone (70mg) which showed negligible incorporation of activity.

Incorporation of [3-^{14}C]Chrysophanol (59)

A solution of [3-^{14}C]chrysophanol (50mg; 7.3 x 10^6 d min.$^{-1}$ m.mol^{-1} in acetone (40ml) was prepared. This solution was fed exactly as above to 10 flasks and harvested. The mycelium was extracted to yield tajixanthone (80mg) but again no signficant incorporation of activity had occurred.

Incorporation of [Methyl-$^2H_3$]Crysophanol (60)

Feeding experiments with this labelled precursor were carried out exactly as [3-^{14}C]chrysophanol (59). Extraction of the dried mycelium yielded tajixanthone (75mg) which by n.m.r. and mass spectral analysis did not show any sign that the labelled precursor had been incorporated.

Incorporation of [Methyl-$^2H_3$]Islandicin (62)

This experiment was also conducted exactly as for [3-^{14}C]islandicin, but again there was no evidence that (62) had been

incorporated to any extent.

Ethyl-3,3-dimethylacrylate

A solution of 3,3-dimethylacrylic acid (20g; 0.2mol), absolute ethyl alcohol (20g; 0.43mol) and concentrated sulphuric acid (4.4ml) in dry benzene (50ml) was gently refluxed for 10 hours. The reaction mixture was poured into excess water (150ml) and the benzene layer separated. The organic layer was washed with saturated sodium hydrogen carbonate solution and water (twice) and dried ($MgSO_4$). Evapouration of the solvent (in vacuo) at 40^{o} gave a pale yellow liquid that was distilled under reduced pressure. The fraction (28.5g) which distilled at 64-66^{o}C (20mm Hg) was collected.

3-Methylbut-2-en-1-ol

To a solution of lithium aluminium hydride (2.5g)in ether 400ml) was added, ethyl-3,3-dimethylacrylate (28.5g; 0.22mol) in dry ether (400ml), during a period of 30 minutes. The mixture was gently refluxed for one hour. A further portion of lithium aluminium hydride (2.5g) was added and the process continued for another hour. The reaction mixture was cooled and water added to it cautiously. The aluminium salts were filtered off, washed with water and ether, the filtrate was extracted with more ether. Evaporation of the dried etheral solution gave an oil (23.5g). The crude product was the distilled under reduced pressure. The fraction (19.0g) which distilled at 55^{o}C (21mm Hg) was collected.

1-Bromo-3-methylbut-2-ene

3-Methylbut-2-en-1-ol (19g; 0.22mol) in dry ether (300ml) was treated with phosphorus tribromide (9ml, 27.5g; 0.10mol) during 30 minutes. The solution was stirred, protected from light and refluxed for four hours. The reaction mixture was cooled, poured on ice water, shaken and extracted with ether (3 x 50ml). The extracts were

combined, washed with saturated sodium bicarbonate solution and water (twice) and dried over sodium sulphate. The solvent was removed and the crude bromide (20.9g) was distilled. The fraction (19.5g) which boils at 28-30°(15mm Hg) was collected. δ H 1.76 (3H, s, $C\underline{H}_3$); 1.81 (3H, s, $C\underline{H}_3$); 4.03 (2H, d, J 8Hz, $C\underline{H}_2$); 5.56 (1H, t, 8Hz, =$C\underline{H}$).

2-(3-Methylbut-2-enyloxy)-benzaldehyde (46)

A mixture of salicylaldehyde (1g; 8.1mmol), 1-bromo-3-methyl-but-2ene (1g; 6.7mmol) and potassium carbonate (1g; 7.2mmol) in dry acetone (15ml) was refluxed for 18 hours. The product was filtered and the solvent evaporated. The oily residue was subjected to preparative thin layer chromatography using 5% ethyl acetate in benzene as developing solvent. The band Rf0.46 was isolated and eluted with chloroform to yield (46) as a yellow oil (692mg; 45%).

νmax ($CHCl_3$) 2920, 2880, 1685 and 1600 cm^{-1}.

δ H 1.72 (3H, s, $C\underline{H}_3$); 1.78 (3H, s, $C\underline{H}_3$); 4.62 (2H, d, J 8Hz, $C\underline{H}_2$); 5.48 (1H, t, J 8Hz, = $C\underline{H}_2$); 6.9-7.8 (4H, m, Ar$\underline{H}$); 10.48 (1H, s, $C\underline{H}O$).

A further band (Rf 0.35) was also isolated and eluted with chloroform to give a solid which on recrystallisation from light petroleum (30-40°) gave 4-hydroxy-4-[2-(3-methylbut-2-enyloxy)]-phenyl-butan-2-one (48) as crystals m.p. 53-54°C. (Found C,70.90; H,7.27. $C_{15}H_{20}O_3$ requires C,70.76; H, 7.49%).

νmax (KBr) 3470,2850, 1670, 1560 cm-1.

δH 1.70 (3H, s, $C\underline{H}_3$); 1.75 (3H, s, $C\underline{H}_3$); 2.10 (3H, s, $C\underline{H}_3CO$); 2.84 (2H, d, J 4Hz, $C\underline{H}_2CO$); 3.53 (1H, d, J 5Hz, O$\underline{H}$, exchangeable); 4.50 (2H, d, J 6Hz, $OC\underline{H}_2CH$=); 5.38 (2H, m, Ar$C\underline{H}O$ and =$C\underline{H}$-); 6.76-7.40 (4H, m, Ar$\underline{H}$).

5-Methoxy-2-(3-methylbut-2-enyloxy)benzaldehyde (47)

A solution of 2-hydroxy-5-methoxy benzaldehyde (1g; 6.5mmol), 1-bromo-3-methylbut-2-ene (1g; 6.7mmol) and potassium carbonate (1g; 7.2mmol) in dry acetone (20ml) was heated under reflux for 18 hrs. the resulting mixture was filtered and the solvent evaporated to furnish an oily residue (2.059g). Purification of the residue by t.l.c. using 10% ether in benzene as eluent, gave two major bands. The band with RfO.72 was the desired product 5-methoxy-2-(3-methylbut-2-enyloxy)-benzaldehyde (47; 643mg; 45%). (Found M^+220, $C_{13}H_{16}O_3$ requires M, 220).

δ_H1.70 (3H, s, $C\underline{H}_3$); 1.76 (3H, s, $C\underline{H}_3$); 3.70 (3H, s, $OC\underline{H}_3$); 4.47 (2H, d, J 7Hz, $OC\underline{H}_2CH$=); 5.40 (1H, t, J 7Hz, =$C\underline{H}$-); 6.74 (2H, m, H-3 and H-4); 7.01 (1H, m, H-6); 10.25 (1H, s, $C\underline{H}O$).

The band with RfO.35 was rechromatographed using 10% acetone in light petroleum (40-60^o) as eluent to give two bands. The band at RfO.45 was eluted to give a solid (5.90mg) which was recrystallised from light petroleum (30-40^o) to give 4-hydroxy-4-[5-methoxy-2-(3-methyl but-2-enyloxy)]-phenylbutan-2-one (51) as crystals m.p. 35-36^o (Found M^+, 278; $C_{16}H_{22}O_4$ requires M, 278).

ν_{max} ($CHCl_3$) 2920, 1710 and 1490cm^{-1}.

δ_H 1.70 (3H, s, $C\underline{H}_3$); 1.76 (3H, s, $C\underline{H}_3$); 2.12 (3H, s, $C\underline{H}_3CO$); 2.80 (2H, m, $C\underline{H}_2CO$); 3.61 (1H, m, $O\underline{H}$, exchangeable); 3.70 (3H, s, $O\underline{H}_3$); 4.47 (2H, d, J 7Hz, $C\underline{H}_2CH$=); 5.36 (1H, m, $CH_2C\underline{H}OH$); 5.40 (1H, d, J 7Hz, $CH_2C\underline{H}$=); 6.74 (2H, m, H-3 and H-4); 7.10 (1H, s, H-6).

The band with RfO.37 was also isolated to give 4-[5-methoxy-2-(3-methylbut-2-enyloxy)]-phenylbut-3-en-4-one as a solid (190mg; 14%) which was recrystallised from benzene, m.p. 126-127^{o}C. (Found M^+ 192 $C_{11}H_{12}O_3$ requires M, 192).

ν_{max} (KBr) 1680, 1650, 1603, 1520 and 1430cm^{-1}

δH 2.38 (3H, s, $C\underline{H}_3$); 3.75 (3H, s, $OC\underline{H}_3$); 6.06 (2H, s); 6.80 (2H, s, H-3 and H-4); 6.38 (1H, d, J 16Hz, =C<u>H</u>); 6.98 (1H, s, H-6); 7.82 (1H, d, J 16Hz, =C<u>H</u>).

<u>3-Methoxy-2-(3-methylbut-2-enyloxy)benzaldehyde</u> (48)

A solution of 2-hydroxy-3-methoxy benzaldehyde (1g; 6.5mmol), 1-bromo-3-methylbut-2-ene (1g; 6.7mmol) and potassium carbonate (1g; 7.2mmol) in dry acetone (20ml) was heated under reflux for 18 hours. The solution was filtered and the solvent evaporated to furnish an oily residue (2.8gr). Purification of the residue by t.l.c. using 5% ethyl acetate in benzene as eluent, gave one major band which was separated and eluted with chloroform to give the desired product 3-methoxy-2-(3-methylbut-2-enyloxy) benzaldehyde (48; 1.15gr; 90%). δH 1.59 (3H, s, $C\underline{H}_3$); 1.72 (3H, s, $C\underline{H}_3$); 3.85 (3H, s, $OC\underline{H}_3$); 4.64 (2H, d, J 8, Hz, $C\underline{H}_2$); 5.50 (1H, t, J 8Hz, =C<u>H</u>); 7.06 (1H, d, J 2Hz, H-6); 7.34 (1H, t, J 2Hz, H-5); 7.36 (1H, d, J 2Hz, H-4); 10.30 (1H, s, C<u>H</u>O).

<u>Cis and Trans 3-Isopropenyl-8-methoxy-chroman-4-ol</u> (57) and (58)

3-methoxy-2-(3-methylbut-2-enyloxy) benzaldehyde (48) (866mg; 3.93mmol) was added to a well stirred suspension of <u>para</u>-toluene sulphonic acid (20mg; 0.1 mmol) in dry benzene (25ml), the mixture was stirred for 72 hrs. The reaction mixture was then filtered and the solvent removed (in vacuo). The oily residue was purified by t.l.c. using 60% ether in light petroleum (30-40%) as developing solvent. The band with RfO.38 was separated, eluted with chloroform to give an oily residue (668mg), which was rechromatographed using 30% ether in light petroleum (30-40^{o}) as eluent. After six elutions two bands were separated. The band with RfO.26 found to be the cis isomer (57) which was recrystallised from ether-light petroleum (30-40^{o}) m.p. 63-65^{o}C. (Found M^{+}220, $C_{13}H_{16}O_3$ requires M,220). (Found C,70.90; H, 7.27.

$C_{13}H_{16}O_3$ requires C,70.76; H,7.49%).

ν_{max} 3520, 1640, 1590 and 1485cm^{-1}.

The band with RfO.30 was removed to give the trans isomer (58) which was recrystallised from ether-light petroleum (30-40^{o}) to give colourless crystals m.p. 82-84^{o}C. (Found C, 70.95; H,7.32. $C_{13}H_{16}O_3$ requires C,70.76; H,7.49). (Found M^{+}220, $C_{13}H_{16}O_3$ requires M,220).

ν_{max} (KBr) 3350, 1645, 1585 and 1485cm^{-1}.

Cis and Trans 3-Isopropenyl-chroman-4-ol (55) and (56)

2-(3-methylbut-2-enyloxy)benzaldehyde (1023mg; 5.4mmol) was added to a well stirred suspension of para-toluene sulphonic acid (20mg; 0.1mmol) in dry benzene (25ml), the mixture was stirred for 10 days at room temperature. The reaction mixture was then filtered and the solvent removed (in vacuo). The oily residue was purified by t.l.c. using 10% acetone in light petroleum (40-60^{o}) as developing solvent. The band with RfO.38 was separated eluted with chloroform to give an oily residue (320mg) which was rechromatographed using 30% etheriin light petroleum (30-40^{o}) as eluent. After four elutions two bands were separated. The band with RfO.34 found to be the cis isomer (54; 14mg).

δH 1.86 (3H, s, C$\underline{H}_3$); 2.60 (1H, m, Hc); 4.10 (1H, s, Hb); 4.29 (1H, m, Ha); 4.70 (2H, m, Hd and Hf); 5.06 (1H, m, He); 6.80-7.30(4H,m,Ar$\underline{H}$).

The band with RfO.40 was removed to give the trans isomer. (56; 100mg). Both products were yellow oils.

δH 1.76 (3H, s, C$\underline{H}_3$); 2.48 (1H, m, Hc); 4.08 (2H, m, Ha and Hb); 4.66 (1H, m, Hd); 4.76 (1H, m, He); 4.92 (1H, m, Hf); 6.70-7.40 (4H,m,Ar$\underline{H}$).

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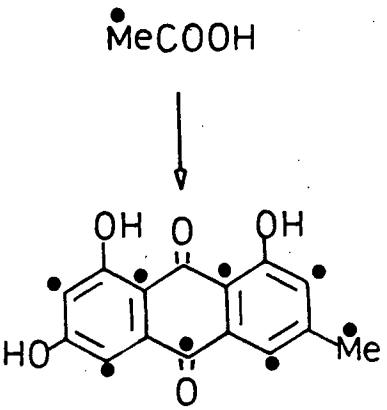
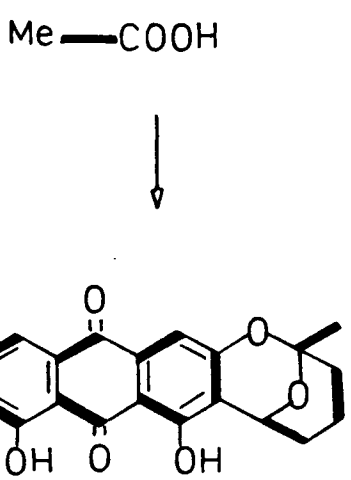
CHAPTER 3

SYNTHESIS OF LABELLED ANTHRAQUINONES AND DIELS-ALDER REACTIONS OF 6-ALKOXY-PYRAN-2-ONES



(1)



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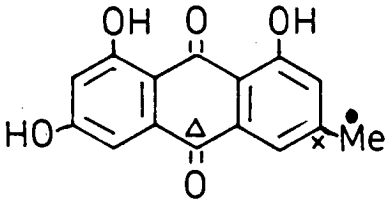
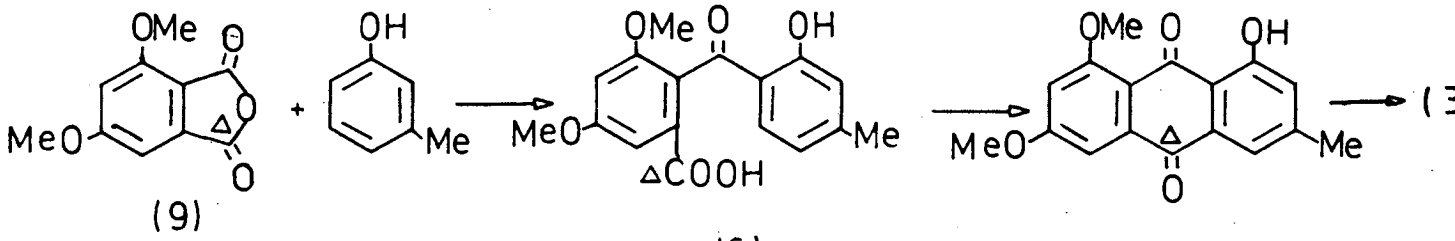


(3) Δ = ^{14}C

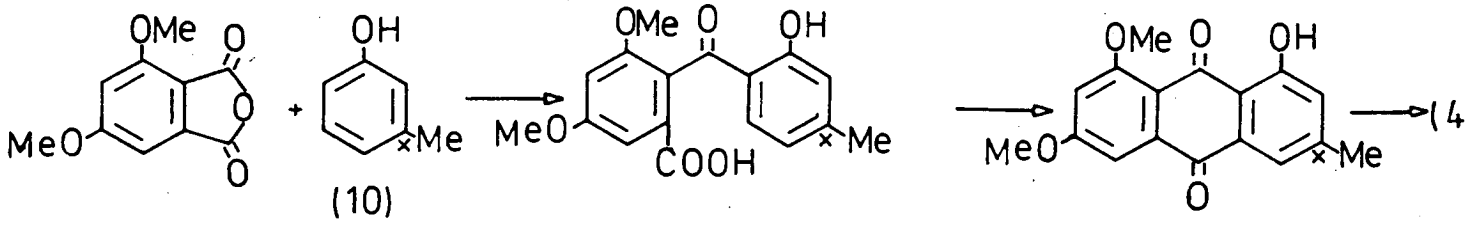
(4) × = ^{14}C

(5) • = ^{14}C



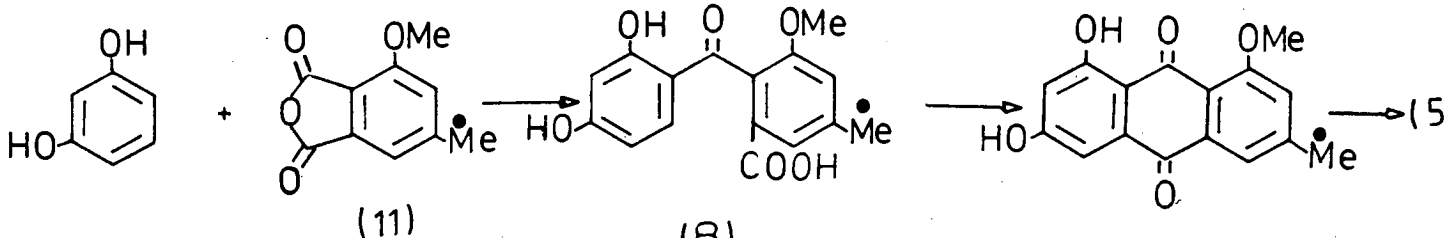
(9)

(6)



(10)

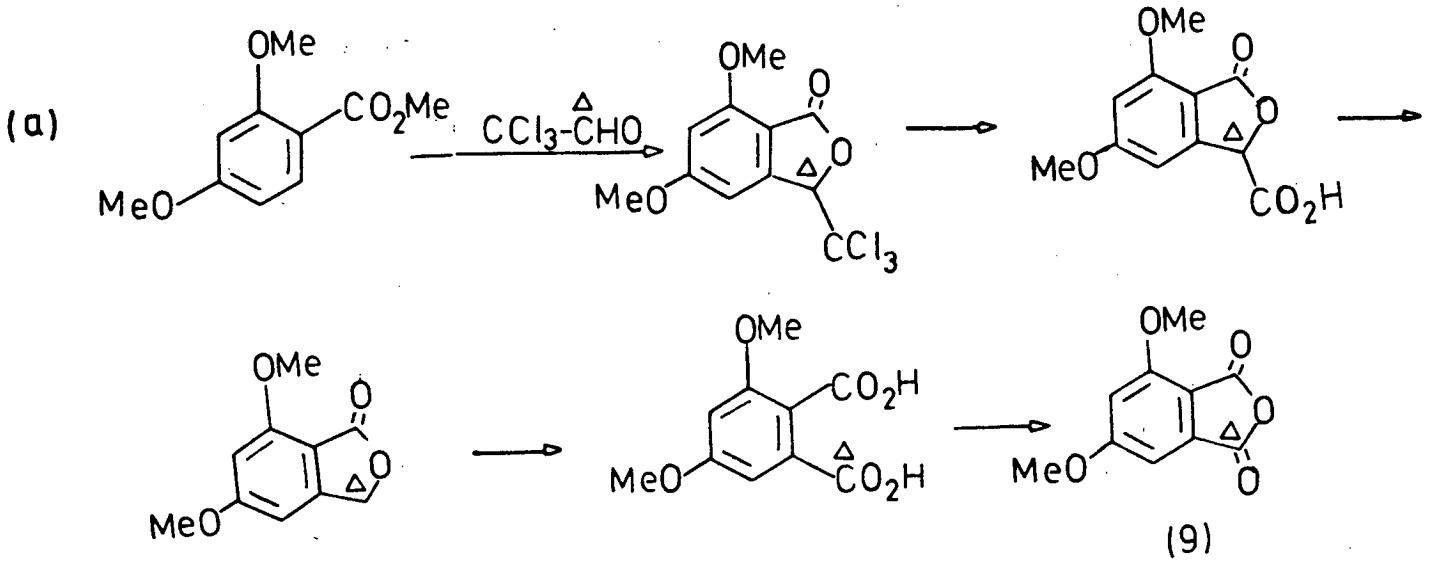
(7)

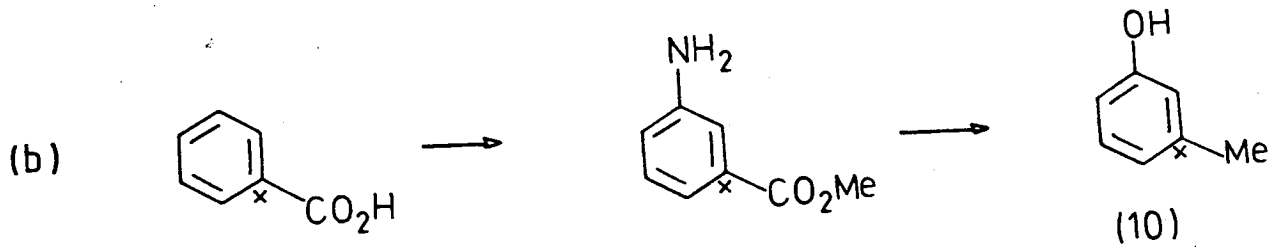


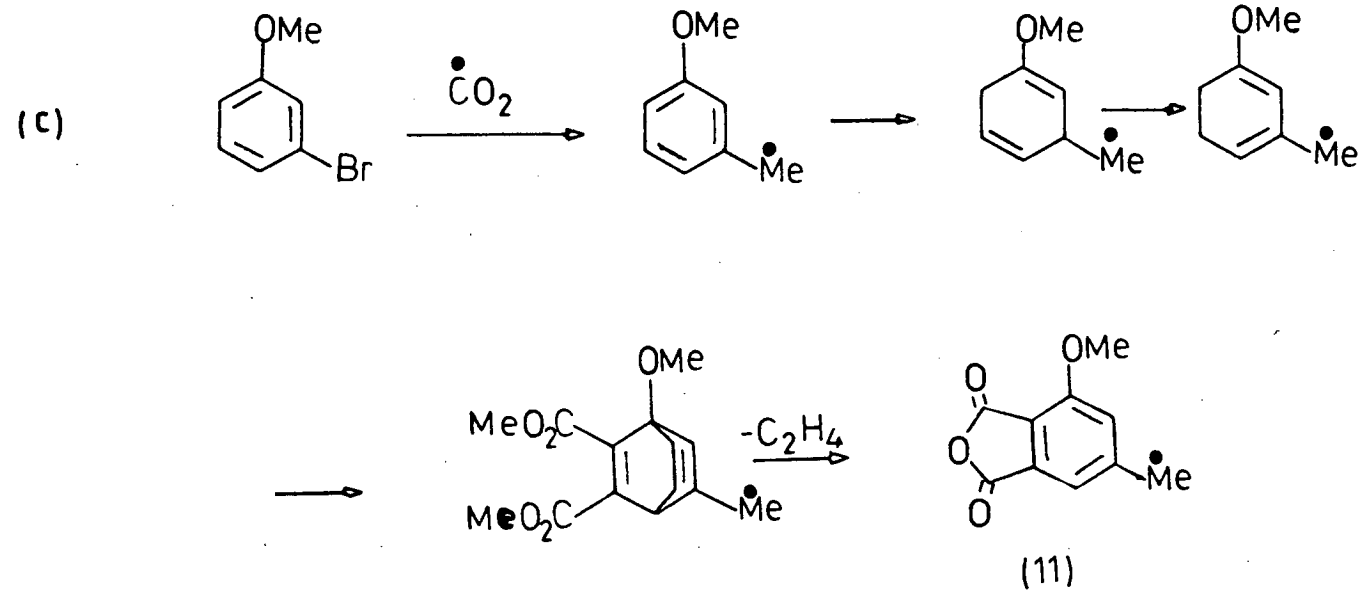
(11)

(8)

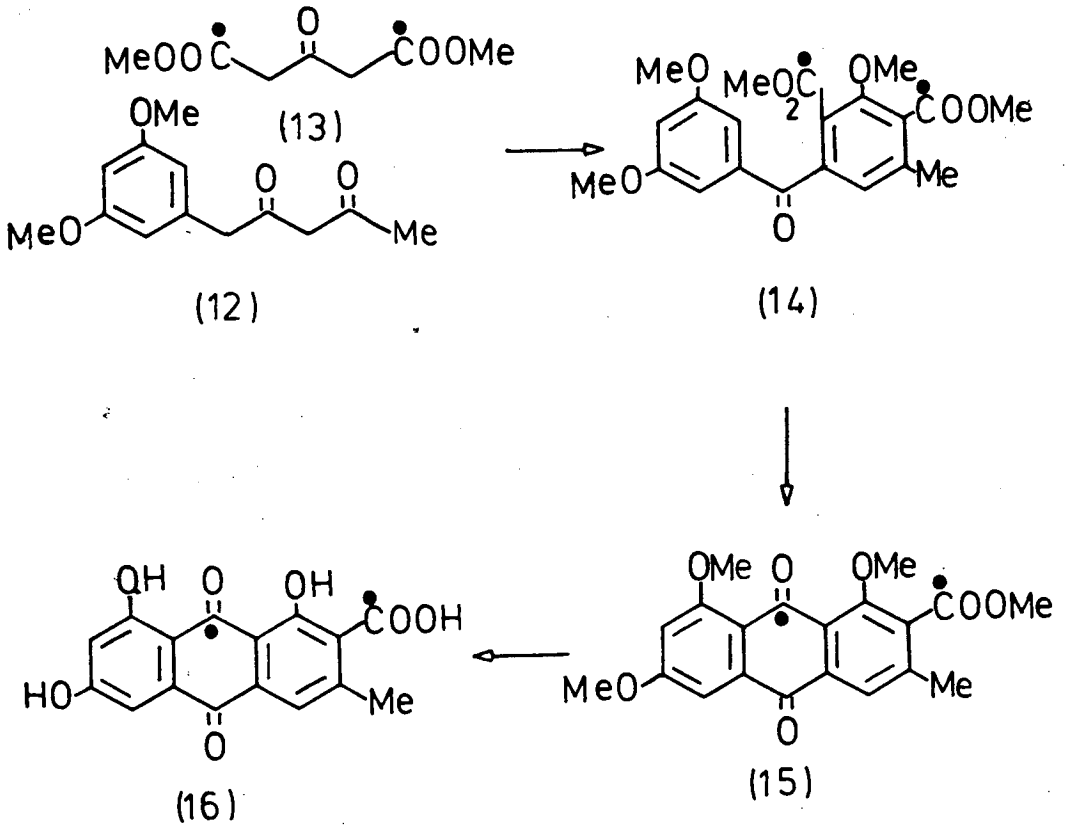
Scheme 1


(a)
OMe
CO2Me
MeO
CCl3-CHO
CCl3
CO2H
(9)


(b)
CO2H
NH2
CO2Me
OH
Me
(10)


(c)
OMe
Br
CO2
Me
MeO2C
-C2H4
(11)

Scheme 2


MeOOC
O
COOMe
(13)
OMe
MeO
O
O
Me
(12)
MeO
MeO2C
OMe
COOMe
MeO
Me
O
(14)
OMe
O
OMe
COOMe
MeO
Me
O
(15)
OH
O
OH
COOH
HO
Me
O
(16)

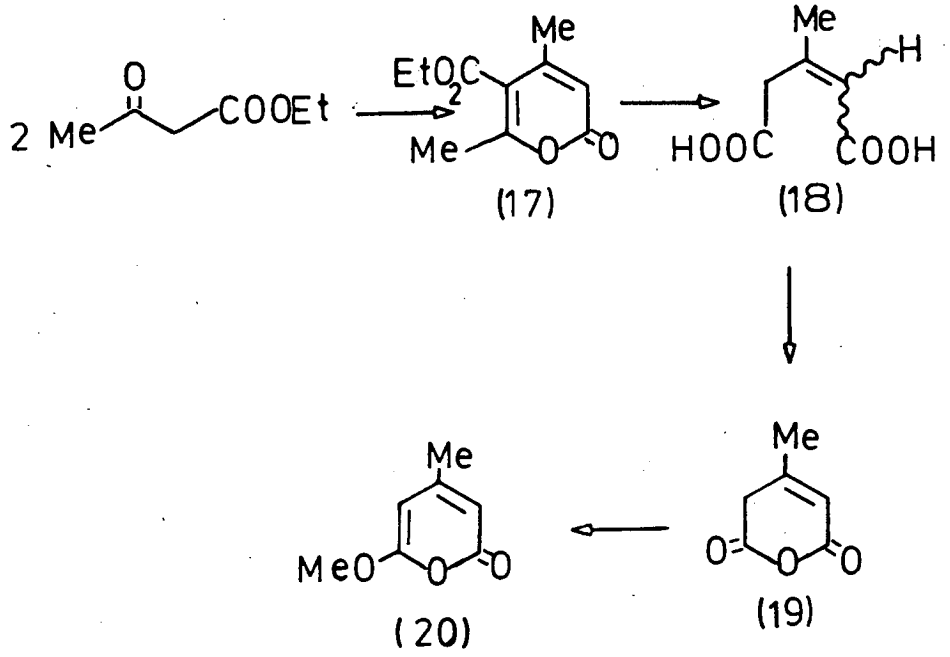
Scheme 3

3. SYNTHESIS OF LABELLED ANTHRAQUINONES

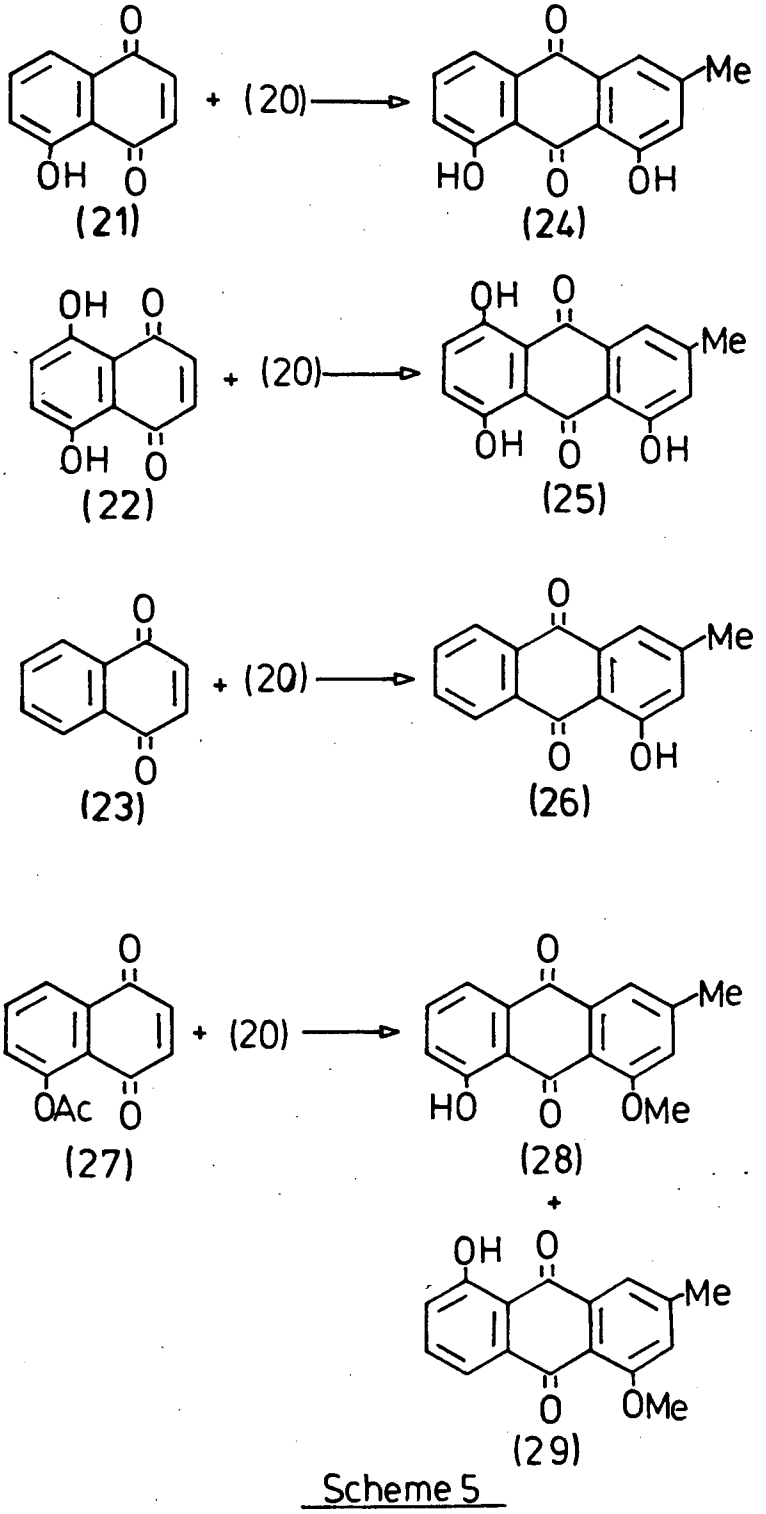
3.1 INTRODUCTION

As many of the precursors needed for the investigation of xanthone biosynthesis are themselves natural products, they can be obtained in a labelled form by feeding labelled acetate to suitable microorganisms and isolation of the final product. For example, labelled emodin(1) and averufin(2) prepared by fermentation of Penicillium islandicum and Aspergillus parasiticus respectively have been used to study the biosynthesis of ergochromes[1] and aflatoxin.[2] However, this method suffers from two main disadvantages; a) the labelling is unspecific; b) the incorporation of labelled acetate is often low. On the other hand, this procedure cannot be considered for precursors which have not been isolated as natural products. Preparation of labelled anthraquinones requires labelling by chemical synthesis or partial synthesis from other uniformly labelled natural products. For example, synthesis of the labelled emodins (3) - (5) with ^{14}C in specific positions has been achieved by the Friedel-Crafts condensations shown in Scheme 1.[3,4] The resulting benzophenone carboxylic acids (6) - (8) on cyclisation in oleum-boric acid and subsequent demethylation furnish the anthraquinones (3) - (5) respectively. The labelled starting materials (9), (10) and (11) were in turn prepared as outlined in Scheme 2. Another example is the synthesis of specifically labelled [^{14}C]endocrocin (16), Scheme 3.[5] In this method condensation between the1-aryl-2,4-diketone (12) and [1,5 -^{14}C]dimethyl-acetone dicarboxylate (13) gave the benzophenone (14) which was further cyclised with polyphosphoric acid to (15). Demethylation then gave the desired product (16).

As is apparent from these examples the methods used to synthesise specifically labelled anthraquinones generally give low


2 Me
O
COOEt
EtO2C
Me
Me
O
O
(17)
Me
H
HOOC
COOH
(18)
Me
O
O
O
(19)
Me
MeO
O
O
(20)

Scheme 4


O
OH O
(21)
+ (20)
Me
HO O OH
(24)
OH O
OH O
(22)
+ (20)
OH O
Me
OH O OH
(25)
O
O
(23)
+ (20)
Me
O OH
(26)
O
OAc O
(27)
+ (20)
Me
HO O OMe
(28)
+
OH O
Me
O OMe
(29)

Scheme 5

overall yields and the labelled starting materials are not readily available.

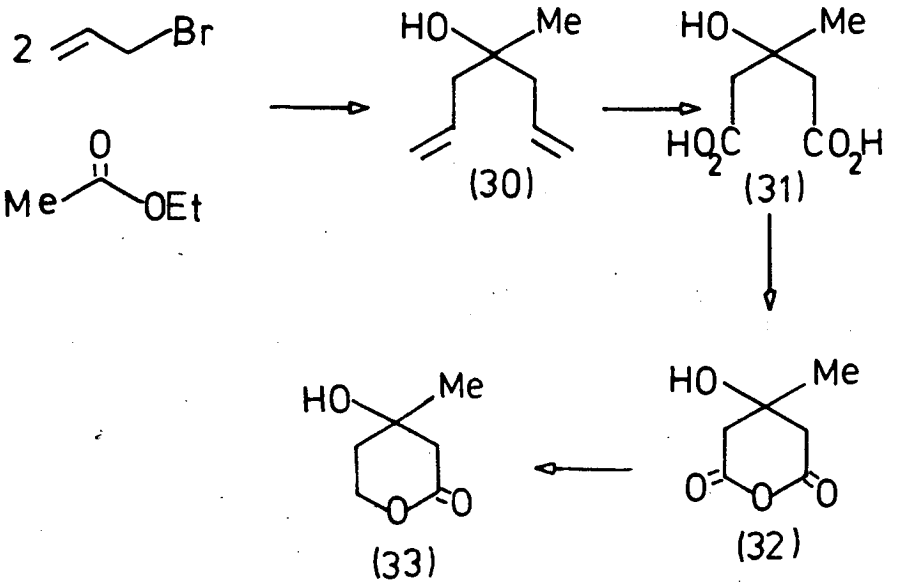
3.2 SYNTHESIS OF CHRYSOPHANOL AND ISLANDICIN

However, a recent method of synthesising anthraquinones developed by Jung and Lowe,[6] provided a potential route to labelled anthraquinones. In this synthetic approach, acid catalysed condensation of ethyl acetoacetate at room temperature provides isodehydroacetic ester (17), Scheme 4. The mechanism of this reaction is believed to be that of an acid catalysed aldol condensation. The cyclic ester is then hydrolysed to glutaconic acid (18). Cyclodehydration of the diacid (18) gives glutaconic anhydride (19) which is trapped in its enolic form by reacting it with diazomethane in ethereal solution to give 6-methoxy-4-methyl- pyran-2-one (20). Diels-Alder addition of (20) which acts as a diene, to a number of naphthoquinones gave after oxidation and demethylation a number of anthraquinones which are shown in Scheme 5.

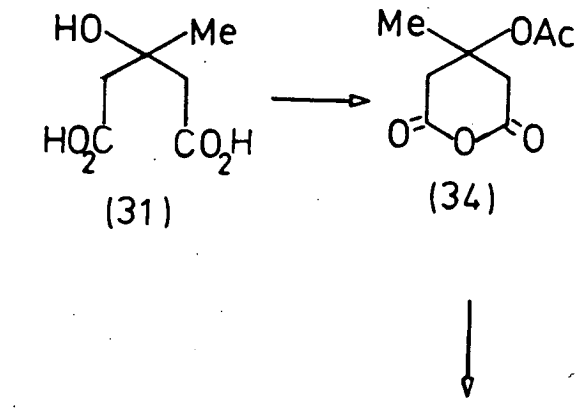
With juglone (21), naphthazarin (22) and 1,4 naphthoquinone (23) itself, good yields of the corresponding anthraquinones, chrysophanol (24), helminthosporin (25) and pachybasin (26) were obtained after demthylation. In the case of juglone only one of the possible isomers is formed, so the reaction is highly regioselective. However, in the case of juglone acetate (27) a mixture of the two possible products chrysophanol methyl ether (28) and ziganein methyl ether (29) are formed in low yield.

On repeating this work we found that the method of synthesising the pyrone (20) outlined in Scheme 4, suffered general disadvantages:

a) the glutaconic acid (18) was obtained as a mixture of cis and trans isomers and was difficult to purify and in cyclising the diacid to the anhydride we confronted



Scheme 6



Scheme 7

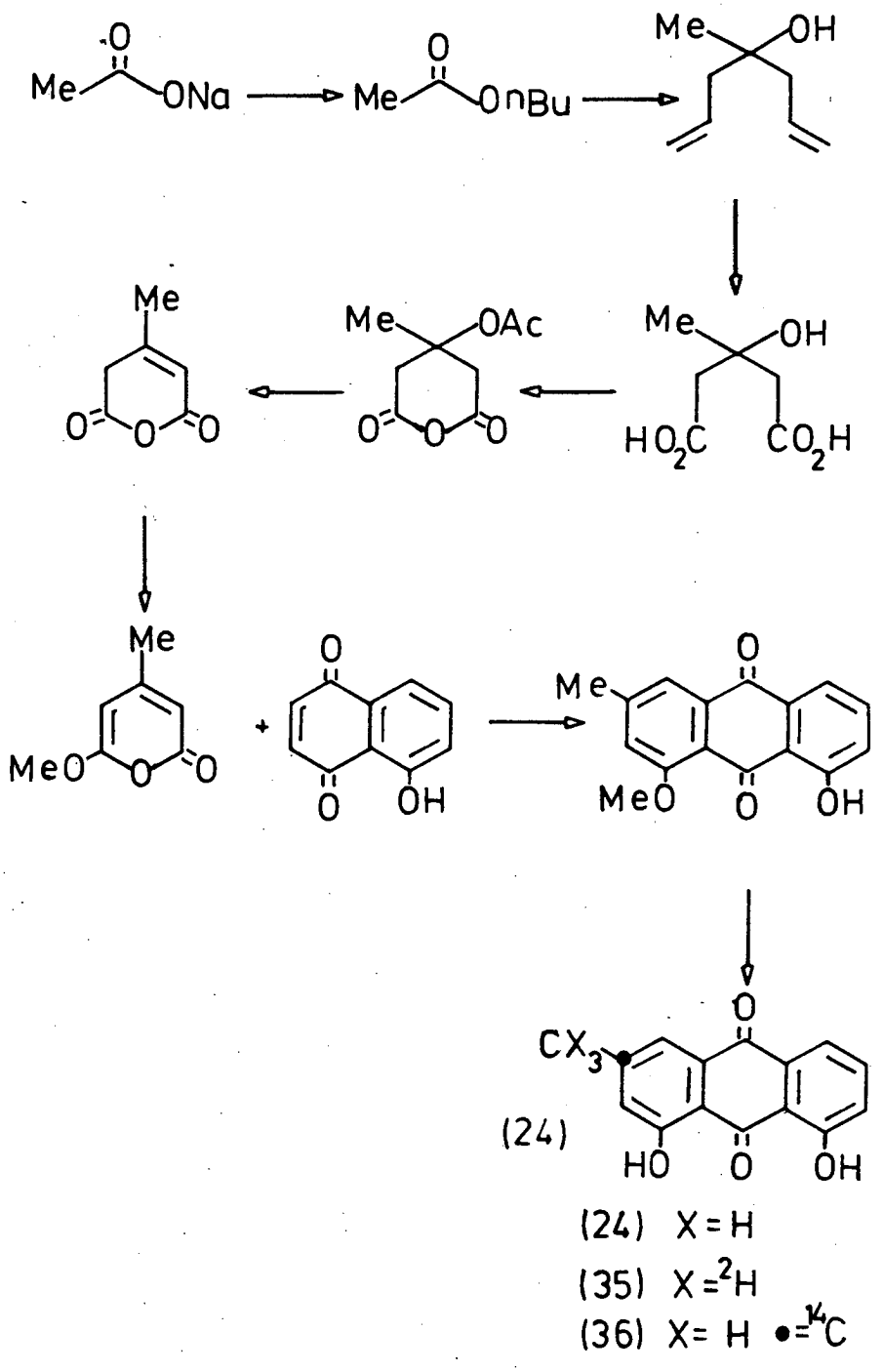
many difficulties doing the reaction, and even when eventually the reaction was carried out the yield was very low.

b) The route is not very convenient for introducing isotopic labels as half the label would be lost in the hydrolysis step.

c) This route cannot be easily adapted to synthesise pyrones with substituents other than methyl.

However, a recently described synthesis[7] of mevalonic acid lactone (Scheme 6) suggested a different route to the pyrone which would be a suitable one for introduction of isotopic label. In this method condensation of one mole of ethyl acetate and two moles of allyl bromide in a Grignard reaction gave the alcohol (30). Subsequent ozonolysis of this alcohol followed by oxidation of the ozonide afforded 3-methyl-3-hydroxyglutaric acid (31). Treatment of (31) with an "excess" of acetic anhydride at room temperature furnished the corresponding anhydride (32). Reduction of this anhydride by sodium borohydride in propan-2-ol followed by acidification and extraction gave almost pure (RS)-mevalonic acid lactone (33) in high yield (75.6%). By altering this route we have been able to obtain the pyrone (20) in good yield as shown in Scheme 7. In our altered method cyclodehydration of the diacid (31) was carried out using acetyl chloride which gave 3-acetyl-3-methyl glutaric anhydride (34). The anhydride was then pyrolysed to 3-methylglutaconic anhydride (19) in quantitative yield. The anhydride (19) is then converted to (20) as described above.

The low boiling point of ethyl acetate (77^{o}C) is something of a disadvantage in using this ester to prepare labelled pyrone (20). In addition, isotopically labelled acetate is more readily available[8]



(24)

(24) X = H

(35) X = ^{2}H

(36) X = H • = ^{14}C

Scheme 8

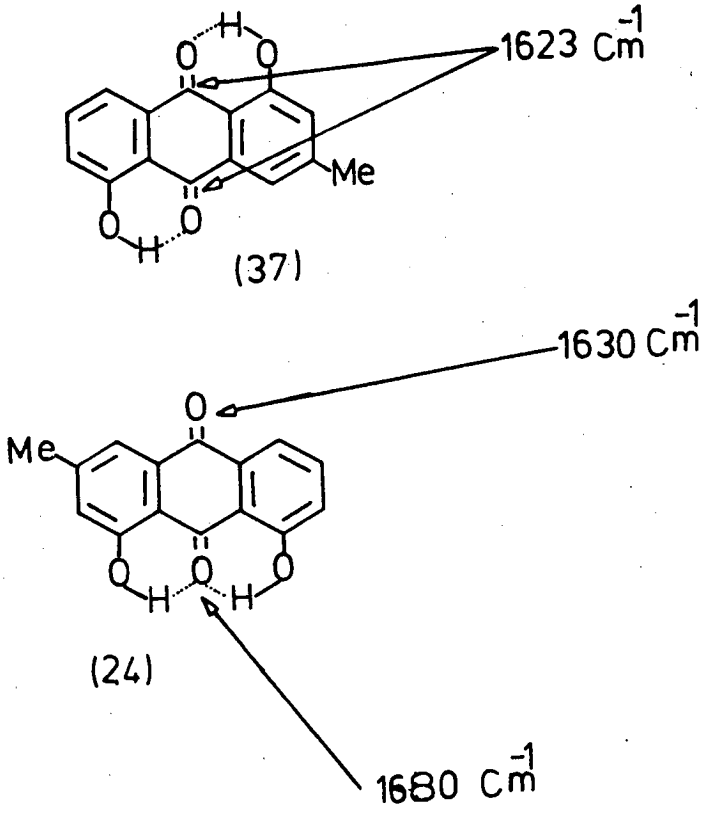
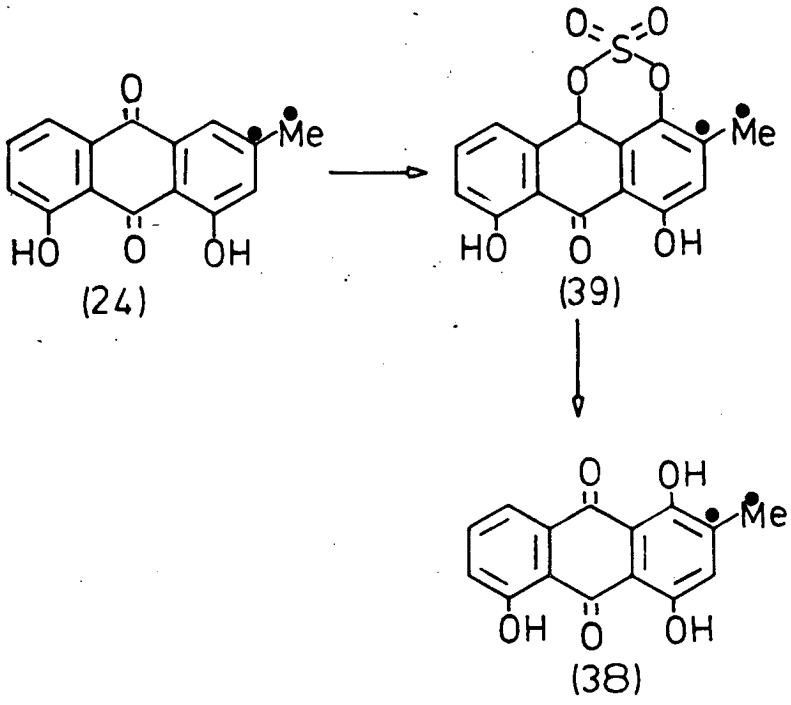


Figure 1 Infra red absorptions for the quinonoid carbonyls in ziganein (37) and chrysophanol (24).

with a variety of labels (^{13}C, ^{2}H, ^{14}C, ^{3}H) and is more conveniently handled as the sodium salt. The sodium salt can be converted into the ethyl ester in high yield by treatment with an excess of triethyl phosphate.[9] However separation of the ethyl acetate from the triethyl phosphate requires careful fractional distillation. Thus it was decided to use the higher boiling n-butyl acetate (b.p. 127°C). The latter was prepared by the method described by Ropp.[9] In this method sodium acetate is heated with n-butyl phosphate to furnish pure n-butyl acetate. This alteration did not adversely affect the Grignard reaction but, in fact, slightly improved the yield of the resulting alcohol.

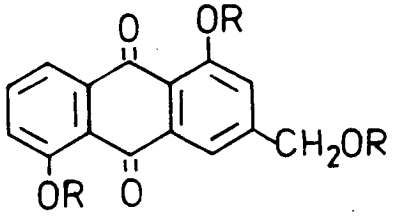
For convenience the whole sequence of the reactions leading to the labelled chrysophanol are summarised in Scheme 8. In this way [methyl-$^{2}H_3$]- and [3-^{14}C]chrysophanol (35) and (36) respectively have been prepared from sodium [2-$^{2}H_3$]acetate and sodium [1-^{14}C]acetate. The n.m.r. spectrum of (35) shows almost complete deletion of the signal representing the methyl group in the unlabelled chrysophanol spectrum. As was mentioned above, the Diels-Alder reaction leading to chrysophanol is highly regioselective and the yield of the other isomer ziganein (37) is very low, but successive experiments and collecting ziganein methyl ether samples provided enough material for the demethylation reaction to be carried out. The ziganein obtained has a higher melting point (205-206°C) compared with chrysophanol (197-198°C); its infra-red spectrum shows a strong absorption at 1623 cm^{-1} compared with that of chrysophanol, which shows two bands at 1630 and 1680 cm^{-1} due to the chelated and non-chelated carbonyls respectively. This is a good indication that in ziganein (37) each carbonyl is chelated to one hydroxyl as shown in figure 1.

Chrysophanol (24) could be converted to islandicin (38) by the



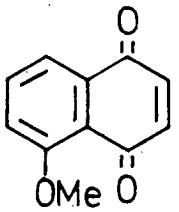
(24) (39) (38)

Scheme 9

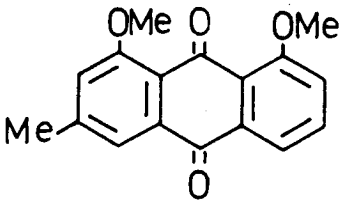


(40) R=H

(41) R=Ac



(42)



(43)

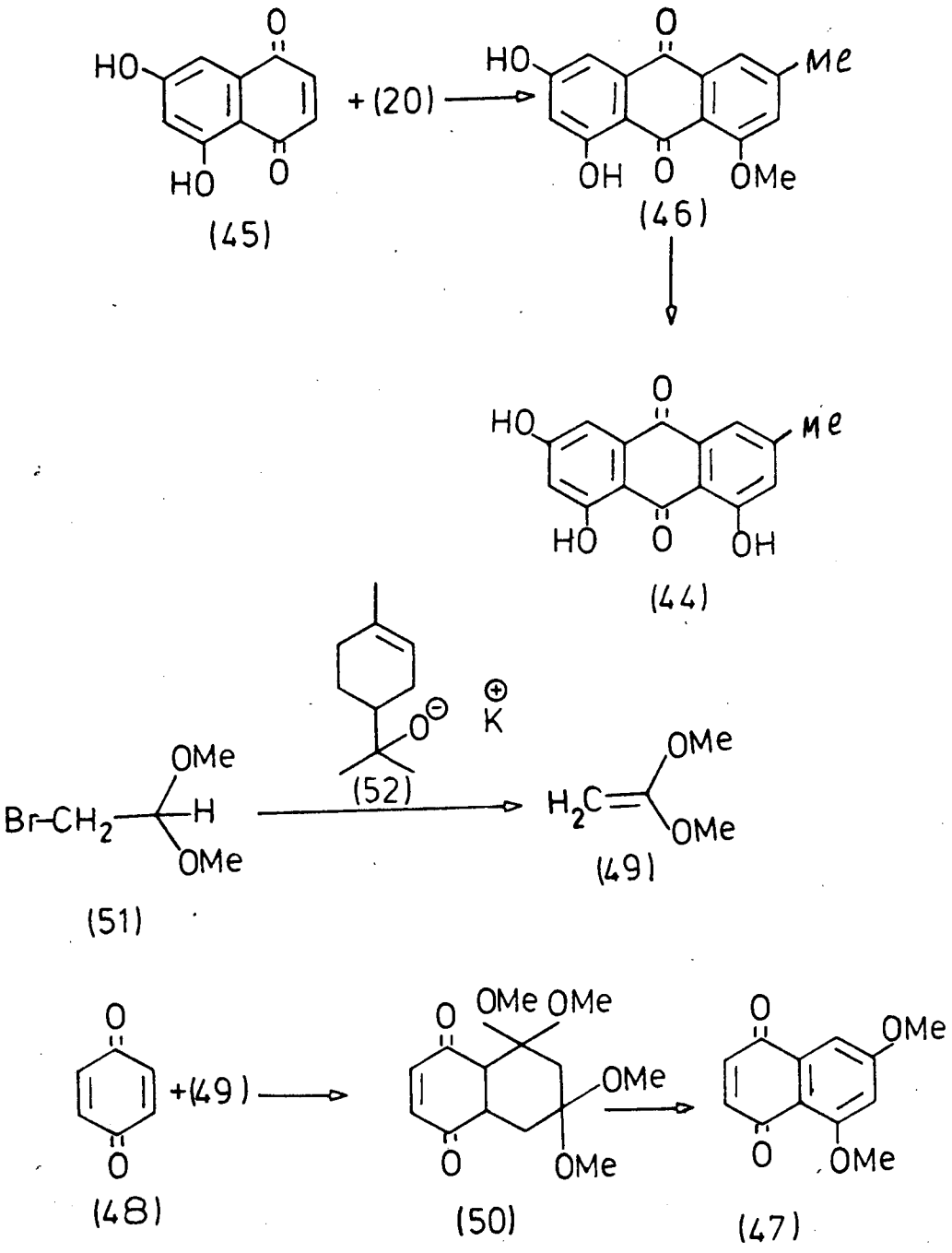
method described by Cameron.[10] In this method 65% oleum serves a hydroxylating agent in the presence of boric acid. The mechanism of this reaction was suggested to be the formation of carbon oxygen linkage at the 4 position as shown in Scheme 9. Subsequent treatment of the intermediate (39) with alkali gives a crude product that after chromatography on silica gel furnishes islandicin (38) in over 50% yield. However, we found that if the crude reaction product is acetylated prior to chromatography the islandicin triacetate is easier to separate from the unreacted chrysophanol (now in the form of its diacetate). Deprotection of this product by refluxing with methanolic hydrochloric acid not only gives islandicin in a purer form but in a higher yield (60%).

When similar hydroxylation of 1,5-dihydroxy-3-methylanthraquinone (ziganein) was investigated, the reaction was very slow. After ca two months at room temperature the reaction mixture showed substantial conversion into a single yellow product (40) which was characterised as its triacetate (41). The appearance of a two-proton resonance at 4.57 p.p.m. in the ^{1}H n.m.r. spectrum of (41) suggested oxidation of the original methyl group to hydroxymethyl had occurred.

The Diels-Alder method of synthesising anthraquinones gives a higher yield than the Friedel - Crafts process, not only because both bonds are formed in one step, but also because of the milder conditions. The reactivity of the juglone methyl ether (42) towards the pyrone (20) was also examined but the result showed that under similar conditions juglone methyl ether (42) is far less reactive than juglone itself, only ca 5% of chrysophanol dimethyl ether (43) was obtained.

3.3 ATTEMPTED SYNTHESIS OF EMODIN

Emodin (44) is also thought to play an important role as an

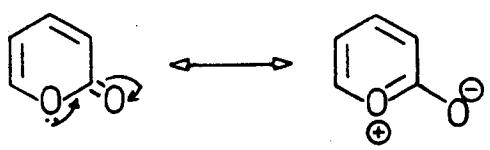

HO
O
HO
O
(45)
+(20)
HO
Me
OH
O
OMe
(46)
HO
Me
HO
O
OH
(44)
OMe
Br-CH2
H
OMe
(51)
O⊖
K⊕
(52)
OMe
H2C
OMe
(49)
O
O
(48)
+(49)
OMe OMe
O
OMe
O
OMe
(50)
O
OMe
O
OMe
(47)

Scheme 10

intermediate in the biosynthesis of some fungal metabolites.[11] To synthesise emodin via the above route 5,7 dihydroxy-1,4-naphthoquinone (45) was required to react as dienophile in a Diels-Alder reaction with pyrone (20) as outlined in Scheme 10. If successful, demethylation of the product (46) would give a potential route to the total synthesis of labelled emodin.

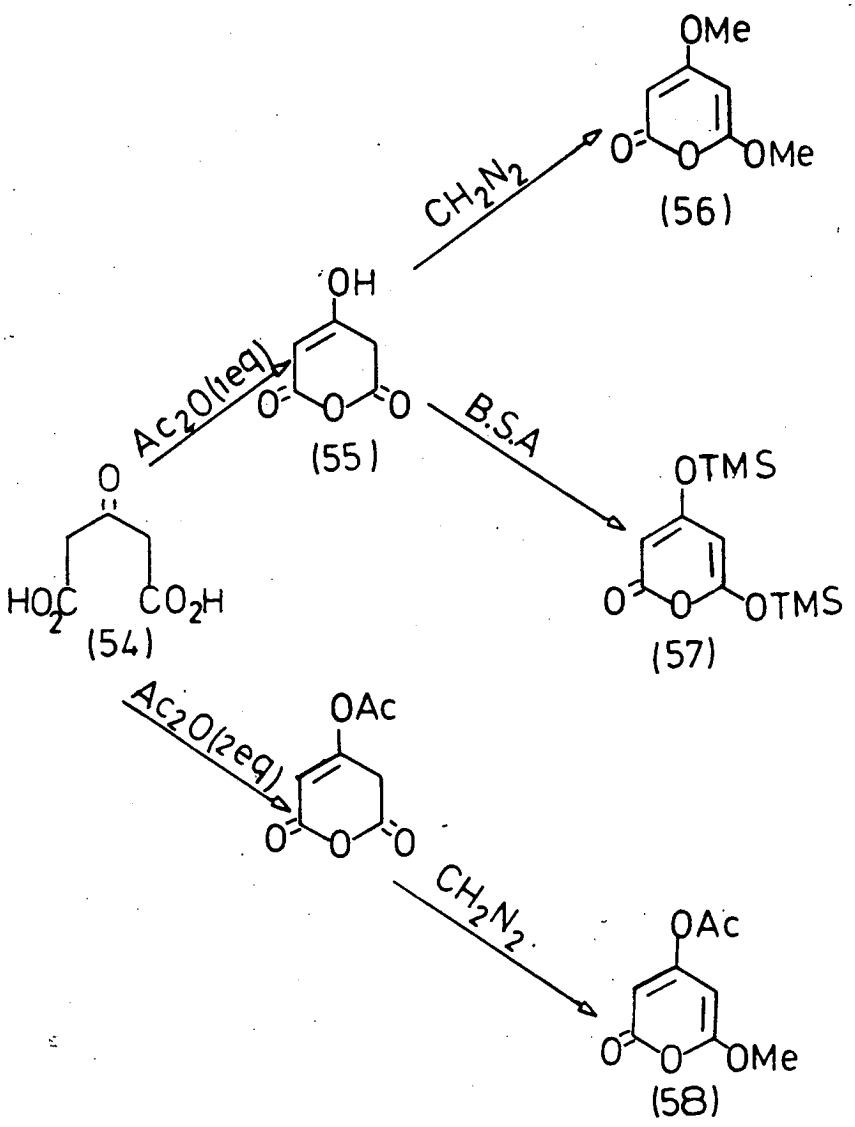
The literature routes to 5,7-dihydroxy-1,4-naphthoquinone itself appeared to be low yielding and involve expensive starting materials.[12] However, a number of different synthetic routes to 5,7-dimethoxy-1,4-naphthoquinone (47) existed; the most convenient seemed to be that of Cameron.[13] In this procedure, Scheme 10, the 1:2 addition of benzoquinone (48) with 1,1-dimethoxyethene (49) in dimethyl sulphoxide furnishes a high yield of the desired naphthoquinone (47). Not only does the reaction take place under very mild conditions (room temperature) but the aromatisation step simply proceeds during the purification of the initial product (50) by t.l.c. on silica gel. 1,1-Dimethoxyethene (49) was prepared by dehydrohalogenation of bromoacetaldehyde dimethyl acetal (51) using the potassium salt of α-terpineol (52) as base.

However, 5,7-dimethoxynaphthoquinone prepared by the above method failed completely to add to the pyrone (20), possibly because the former is deactivated by the electron withdrawing effect of the methoxyls. To overcome this problem it was decided to demethylate the naphthoquinone (47) to give 5,7-dihydroxy-1,4-naphthoquinone (45) and examine its Diels-Alder addition to (20). However all the demethylation attempts using inter alia 48% hydrobromic acid, anhydrous aluminium chloride, in both benzene and nitrobenzene and trimethylsilyl iodide[14] resulted in intractable reaction mixtures and failed to deprotect the naphthoquinone (47), possibly because the two

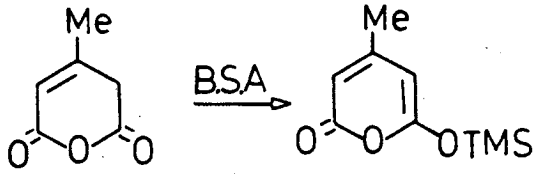


(53)

Figüre 2



Scheme 11



(19) (59)

Scheme 12

positions adjacent to the quinone carbonyls are susceptible to nucleophilic attack.

3.4 FURTHER INVESTIGATION OF DIELS-ALDER REACTIONS OF 6-ALKOXY-PYRAN-2-ONES

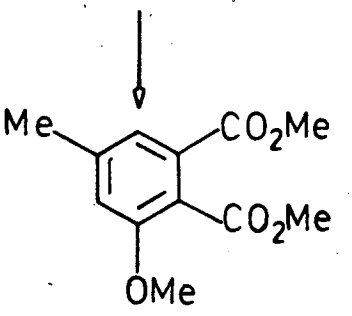
Pyran-2-ones (53) might be expected to be aromatic to some extent since the structure can be written in two canonical forms, figure 2. Since oxygen is more electronegative than nitrogen, the lone pair will be more localised and so pyran-2-one will not be expected to be as aromatic in character as its nitrogen containing analogues.[15] There is some evidence to support this aromaticity, e.g. dipole moment studies and Raman spectroscopy.[15] Magnetic susceptibility measurements,[16] however, indicate little or no aromatic character. This is firmly backed up by the ease with which pyran-2-ones can undergo Diels-Alder reactions. In Diels-Alder reactions pyran-2-ones generally behave as dienes with the elimination of the lactone group as CO_2. The presence of a bulky substituent at the 6-position of pyran-2-ones can inhibit cycloadditions for steric reasons. Finally, Diels-Alder reactions of pyran-2-ones with a wide variety of dienophiles has proved to be highly stereospecific and leads (in most cases) exclusively to one product.[17]

3.4.1 Reactions of 6-Alkoxy-4-methylpyran-2-ones with Different Dienophiles

Since, as has been shown before, 6-methoxy-4-methyl-pyran-2-one (20) is acting as a diene in the Diels-Alder reactions, it seemed appropriate to examine the reactivity of this pyrone and its analogue 6-silyloxy-4-methylpyran-2-one (59) towards a number of frequently used dienophiles.

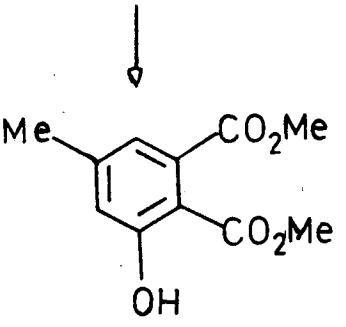
The reactivity of a number of 4,6-dioxypyran-2-ones (56)-(58) in Diels-Alder process has recently been described.[18] These were

(20)



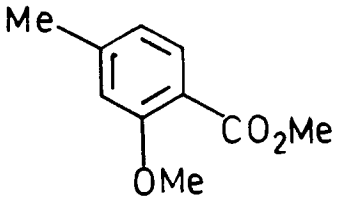
(60)

(59)



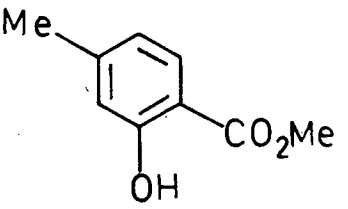
(61)

(20)



(62)

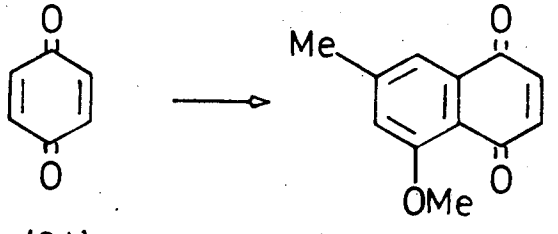
(59)



(63)

Scheme 13

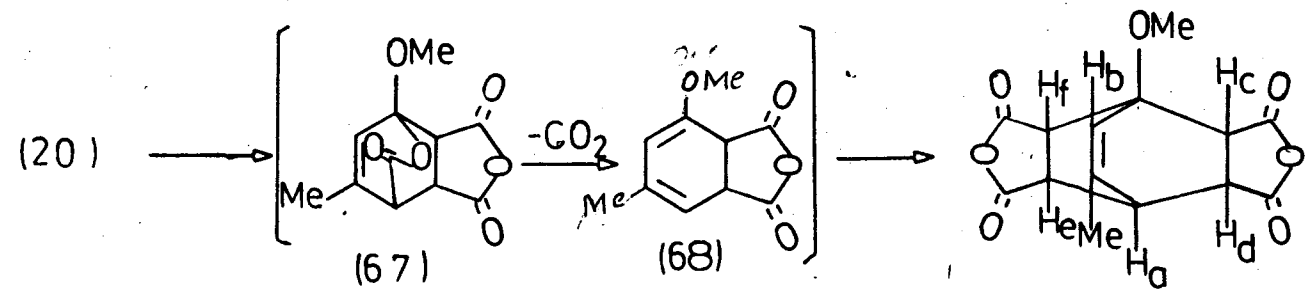
(20) +

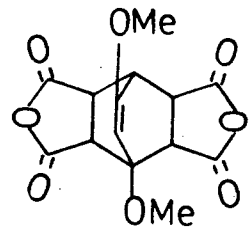


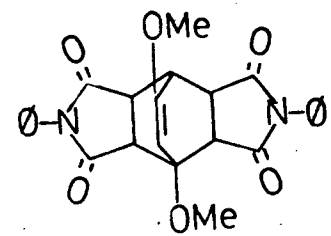
(64)

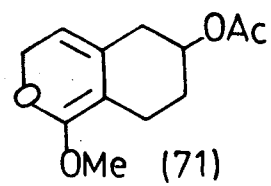
(65)

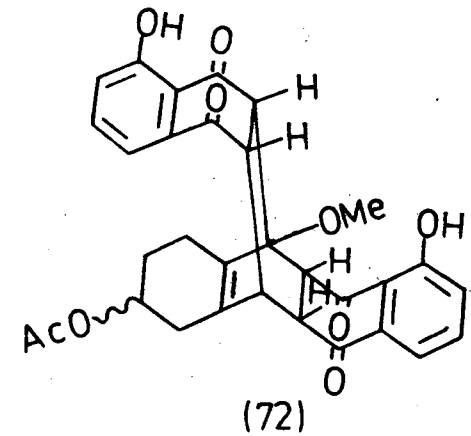
Scheme 14


(20)
OMe
Me
O
-CO2
(67)
(68)
Hf
Hb
Hc
He
Me
Ha
Hd
(66)


OMe
OMe
(69)


OMe
Ø-N
N-Ø
OMe
(70)


OAc
OMe
(71)


OH
H
H
OMe
OH
AcO
(72)

Scheme 15

TABLE 1 100 MHz ^{1}H n.m.r. spectrum of the maleic anhydride adduct (6b).

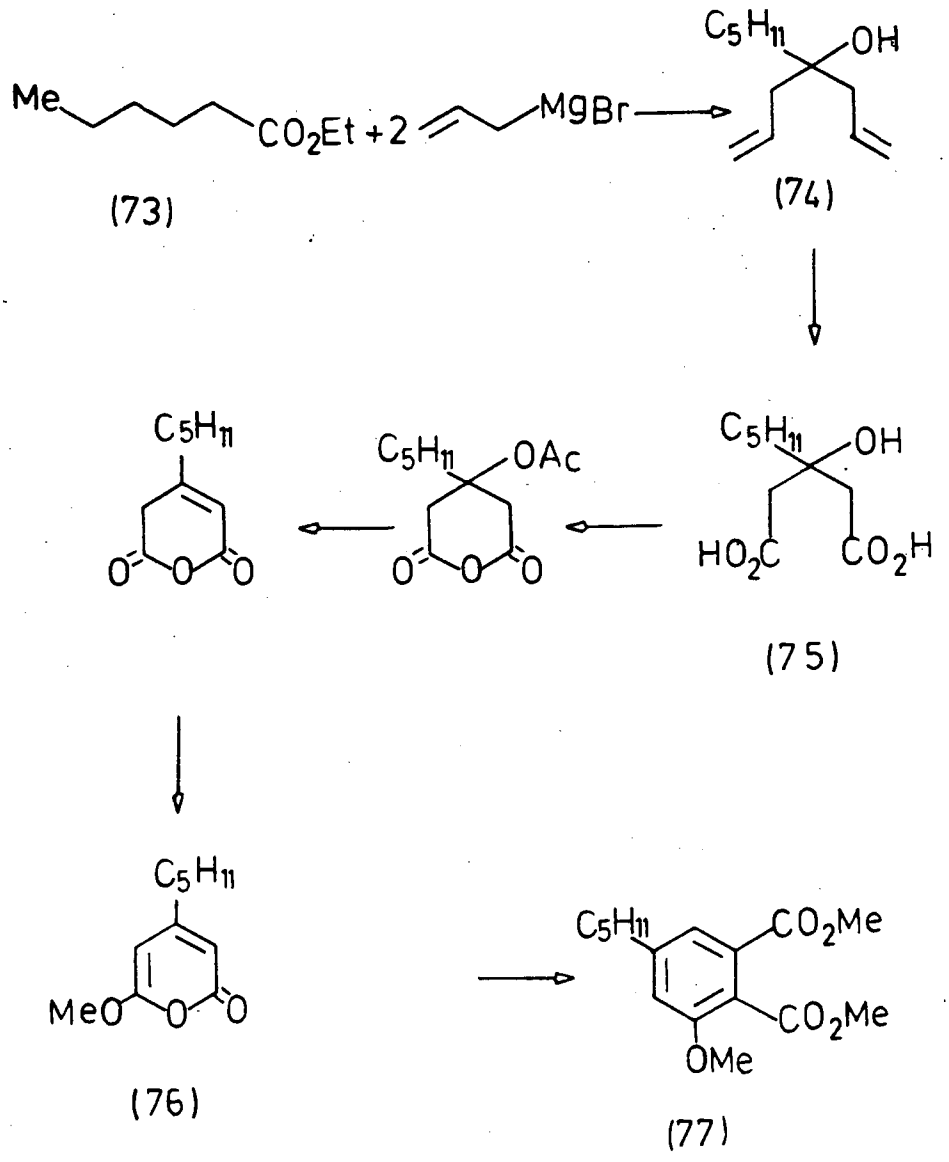
Hydrogens	H(p.p.m.)	No. of protons	Multiplicity (J in Hz)
Me	1.78	3	d (2.0)
Ha	3.45	1	td (9.3, 3.2)
OMe	3.65	3	s
Hd, He	3.86	2	dd (9.3, 9.0)
Hc, Hf	4.10	2	d (9.0)
Hb	5.84	1	m (3.2, 2.0)

obtained as shown in Scheme 11 from acetone dicarboxylic acid (54). In particular, treatment of the anhydride (55) with bis-trimethyl silyl acetamide (B.S.A.) gave the silyloxy pyrone (57). Similar treatment of 3-methylglutaconic anhydride (19) gave the silyloxy pyrone (59) in quantitative yield.

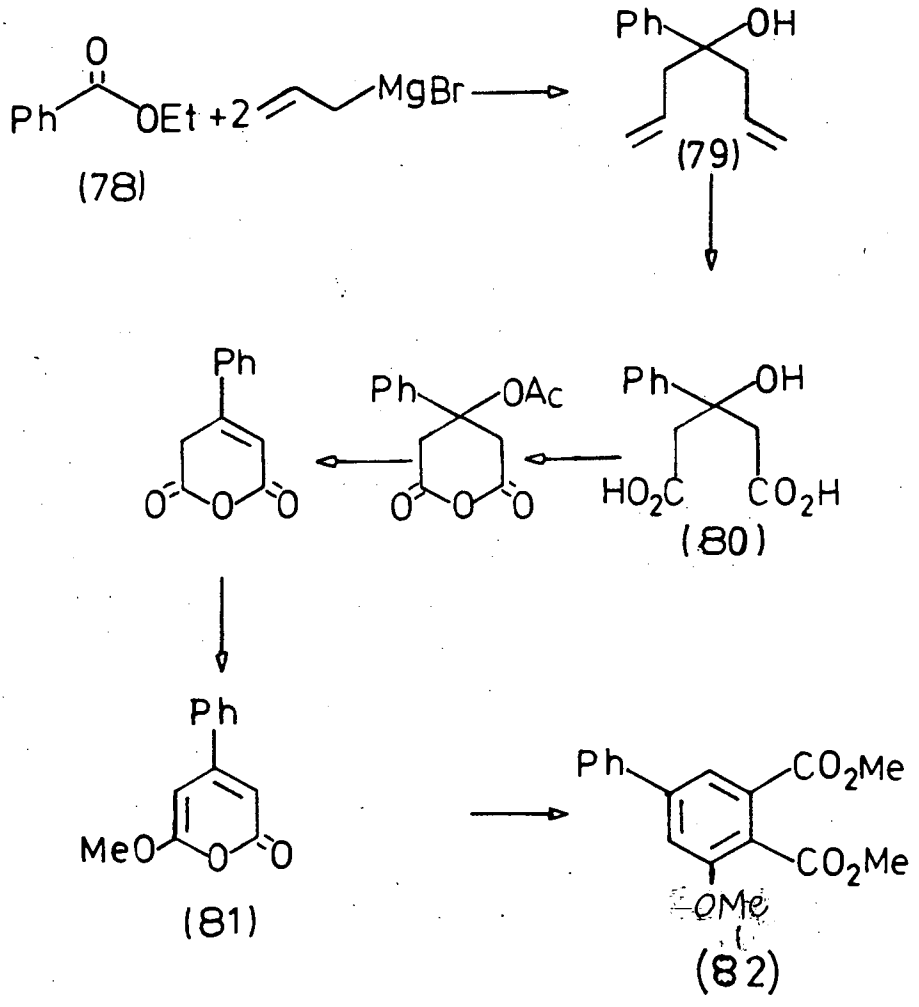
Both pyrones (20) and (59) reacted with acetylenic dienophiles as shown in Scheme 13 to give good yields of products. Thus with dimethyl acetylenedicarboxylate the products (60) and (61) respectively were isolated after work up in ca 70% yield. Pyrone (20) reacted with methyl propiolate to give an equimolar mixture of (62) and (63). The demethylation appears to be occurring on t.l.c. during work up. The silyloxy pyrone (59) however gave a very high yield of (63) under the same conditions. Interestingly, only one regioisomer could be detected with methyl propiolate.

Unlike (20) the silyloxy pyrone (59) did not react with juglone. Also (20) but not (59) reacted with p-benzoquinone (64) to give the naphthoquinone (65). No further addition of (20) to (65) occurred, which is not surprising as juglone methyl ether (42) gives only a very low yield of anthraquinone product under much more forcing conditions.[6]

With maleic anhydride the methoxy pyrone (20) gave a highly crystalline 2:1 adduct which has been assigned structure (66) (Scheme 15) on the basis of its spectroscopic properties. The mass spectrum gave a molecular ion at M^+= 292 corresponding to a molecular formula $C_{14}H_{12}O_7$. The infra red spectrum showed absorptions at 1860 and 1800 cm^{-1} due to anhydride carbonyls. The ^1H n.m.r. spectrum is summarised in Table 1 and showed the presence of an olefinic methyl (1.78p.p.m.) coupled to a multiplet at 5.84 p.p.m. On irradiation of the olefinic methyl this multiplet collapsed to a doublet (J 3Hz). The residual


Me
CO2Et +2
MgBr
C5H11
OH
(73)
(74)
C5H11
OH
HO2C
CO2H
(75)
C5H11
OAc
C5H11
MeO
(76)
C5H11
CO2Me
CO2Me
OMe
(77)

Scheme 16

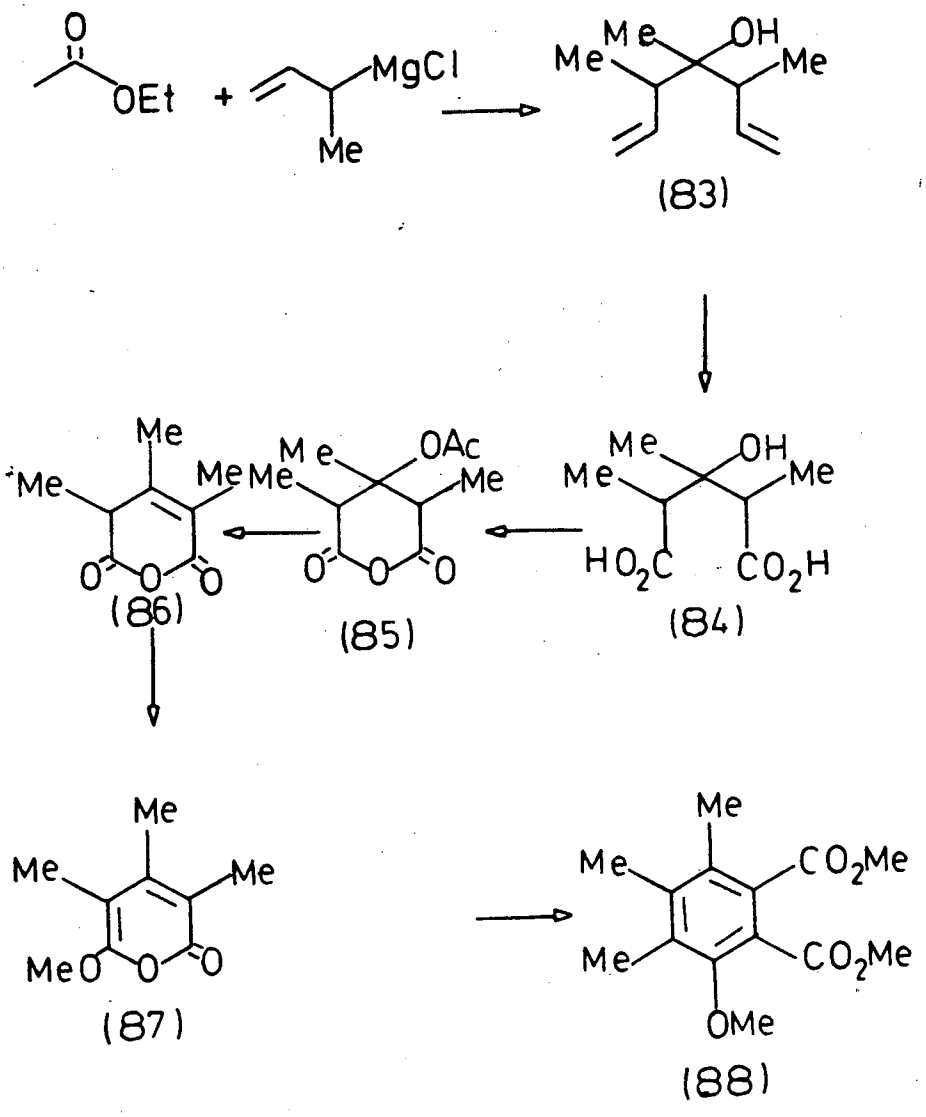

Ph
O
OEt
+2
MgBr
(78)
Ph
OH
(79)
Ph
OAc
Ph
OH
HO2C
CO2H
(80)
Ph
MeO
(81)
Ph
CO2Me
CO2Me
OMe
(82)

Scheme 17

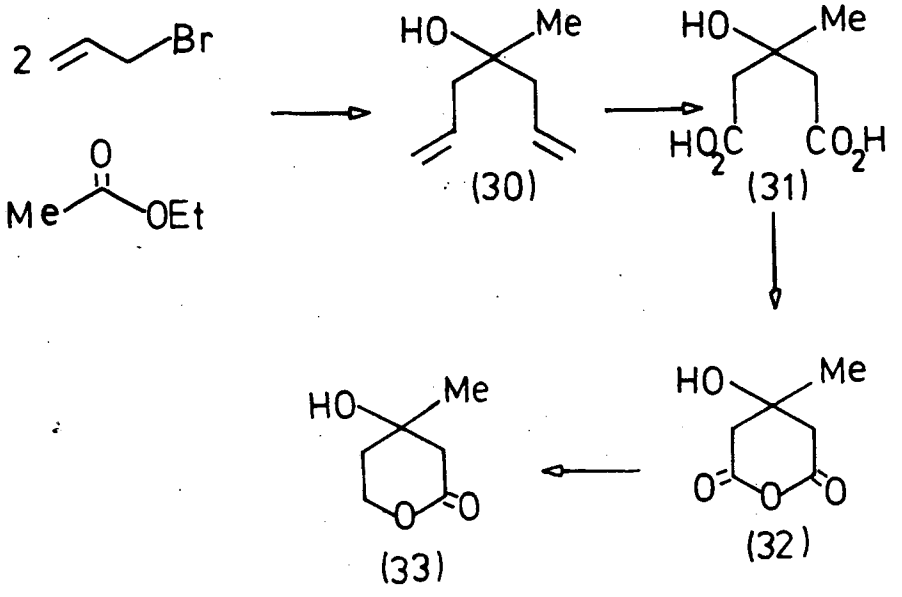
coupling was due to an allylic coupling to the bridgehead hydrogen (Ha) which appears as a triplet of doublets at 3.45 p.p.m. H_a is further coupled to H_d and H_e which appear as a doublet of doublets at 3.86 p.p.m. further coupled to H_c and H_f which gave rise to a doublet at 4.10 p.p.m. The cycloadduct (66) presumably arises from addition of a second equivalent of pyrone (20) to the cyclohexadiene (68) generated by extrusion of CO_2 from the primary cycloadduct (67) as shown in Scheme 15. Similar 2:1 adducts have been reported. Reaction of 4,6-dimethoxypyrone (56) with either maleic anhydride or N-phenylmaleimide gives rise to compounds (69) and (70) respectively.[18] Reaction of the methoxypyrone (71) with juglone (42) gave rise to a 2:1 adduct which has been assigned structure (72).[19]

3.4.2 Synthesis and Reactivity of Further Substituted Pyrones

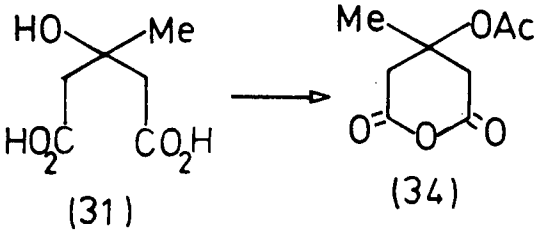
The reactivity observed for pyrones(20) and (59) in the cycloaddition reaction described above provided a stimulus for further investigation of this class of compounds. This part of the research was devoted to determining the flexibility of the synthetic route to substituted pyrones and hence to highly substituted aromatic compounds. A number of substituted pyrones have been synthesised and their reaction with dimethyl acetylene dicarboxylate examined. 6-Methoxy-4-pentylpyran-2-one (76) has been synthesised, as shown in Scheme 16, starting with ethyl hexanoate (73). Reasonable yields were obtained throughout the synthetic sequence and no problems were encountered with the individual reactions. Pyrone (76) was obtained as a low melting solid (m.p. 28-30^{o}C) which reacted cleanly with dimethyl acetylene dicarboxylate to give dimethyl 3-methoxy-5-pentyl-phthalate (77) in 87% yield. Ethyl benzoate (78) also proved a suitable starting material giving rise as shown in Scheme 17 to 6-methoxy-4-phenylpyran 2-one (81) in good yield. With the exception


OEt
MgCl
Me
Me
OH
Me
Me
(83)
Me
OH
Me
Me
HO2C
CO2H
(84)
Me
OAc
Me
Me
Me
Me
Me
(85)
Me
Me
Me
Me
(86)
Me
Me
Me
MeO
(87)
Me
CO2Me
Me
CO2Me
OMe
(88)

Scheme 18


2
Br
Me
OEt
HO
Me
(30)
HO
Me
HO2C
CO2H
(31)
HO
Me
O
O
O
(32)
HO
Me
O
O
(33)

Scheme 6

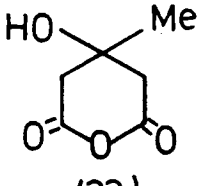

HO
Me
HO2C
CO2H
(31)
Me
OAc
O
O
O
(34)

of the bis-allyl compound (79) all the intermediate products were highly crystalline. Cyclisation of the 3-hydroxy-3-phenylglutaric acid (80) required more time (6 h at 60°C) than its pentyl analogue (75). Reaction of (81) with dimethyl acetylene dicarboxylate again proceeded smoothly to give a good yield (95%) of the biphenyl (82). Ethyl acetate also reacted readily with the Grignard reagent derived from 3-chlorobut-1-ene to give a good yield of 4-hydroxy-3,4,5-trimethylhepta-1,6-diene (83). Ozonolysis gave the corresponding glutaric acid (84) which was cyclised to the acetoxy-anhydride (85). This on heating gave the glutaconic anhydride (86) which reacted with diazomethane to give 6-methoxy-3,4,5-trimethylpyran-2-one (87). The n.m.r. spectrum of compounds (83)-(87) indicated the presence of diastereoisomeric mixtures, and the compounds were non-crystalline probably for the same reason. Although the cycloaddition reaction of pyrone (87) with dimethyl acetylene diarboxylate was given longer time the yield of the fully substituted phthalate (88) was still low (38%). These results show that the reaction of substituted 6-methoxypyran-2-ones with acetylenic dienophiles provides a useful and flexible route to a variety of highly substituted benzenoid compounds.

3.5 REINVESTIGATION OF MEVALONIC ACID SYNTHESIS VIA β-HYDROXY-β-METHYL GLUTARTIC ANHYDRIDE

As has been described above, the synthetic route leading to the pyrone (20) is an adaption of the route devised[7] (Scheme 6) for the synthesis of (R S)-mevalonic acid lactone (33). Since some problems with this synthetic procedure had been reported,[20] it was decided to carry out a reinvestigation of the route. The Grignard reaction to convert ethyl (or n-butyl) acetate to 4-hydroxy-4-methyl-hepta-1,6-diene (30) and its further conversion to the diacid (31)

Figure 3 80 MHz ^1H n.m.r. spectrum of 3-hydroxy-3-methyl- glutaric anhydride (32) in d_6-acetone.



(32)

proceeds without difficulty. However, problems appear to have been encountered in the next step, in which the diacid (31) was converted to the corresponding anhydride (32) by treatment with acetic anhydride. Indeed in a recent study,[20] Lewer and MacMillan, have monitored the reaction of diacid (31) with acetic anhydride by n.m.r. and they reported that the diacid (31) was converted quantitatively to 3-acetoxy-3-methylpentane-1,5-dioic acid anhydride (34) with the desired product (32) being formed only as a transient intermediate. To obtain the anhydride (32), they treated the diacid (31) with N,N-dicyclohexylcarbodi-imide. Accordingly the reaction of diacid (31) with acetic anhydride under a variety of conditions has been studied and to summarise, it has been found that the course of the reaction depends on; (a) the relative concentrations of acetic anhydride and the diacid (31); (b) the reaction temperature; and (c) the batch of diacid (31 used.

As a result of these studies, it appears that the diacid (31) can be converted consistently and in essentially quantitative yield to the desired anhydride (32) provided that less than 20 equivalents of acetic anhydride are used and the reaction temperature is kept below 30°C. In a typical experiment the diacid (31, 405 mg, 2.5 mmol) in acetic anhydride (3.3 ml, 35 mmol) was stirred at 18°C. After 24 h the initial suspension had turned to a clear solution. The excess acetic anhydride was then removed in vacuo to give a white solid shown by n.m.r. (Figure 3) to be essentially pure anhydride (32). On repeating the reaction using the same batch of acid but on double the scale approximately 48 h were required for complete reaction. However, the time taken for complete reaction as judged by the time for a clear solution to be obtained varied markedly with the batch of acid used. In a further experiment the course of the reaction was

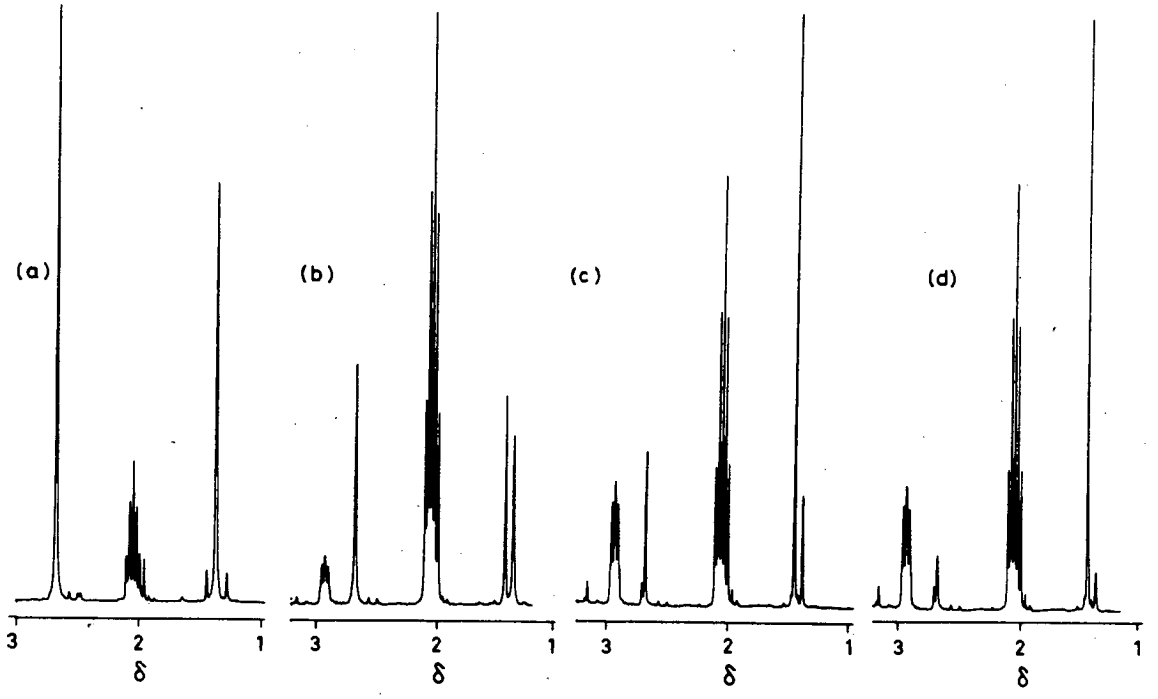
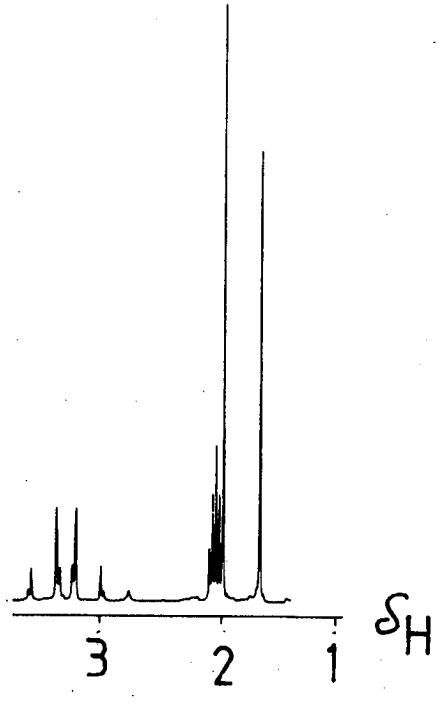
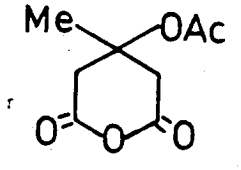


Figure 4 80 MHz ^{1}H n.m.r. spectra of the reaction mixture from 3-hydroxy-3-methylglutaric acid (31) with acetic anhydride (14 equivs.) at room temperature. Samples were taken at (a) t=0, (b) t=2h, (c) t=4h and (d) t=6h. The excess acetic anhydride was removed under pressure and the resulting white solid was dissolved in d_6-acetone.





(34)

Figure 5 80 MHz H n.m.r. spectrum of 3-acetoxy-3-methylglutaric anhydride (34) in d_6-acetone.

monitored by withdrawing small aliquots of the reaction mixture, removing the acetic anhydride immediately *in vacuo*, and determining the 80 MHz n.m.r. spectrum. As shown in Figure 4 the acid was converted smoothly and cleanly to anhydride (32) with no trace of the acetate (34). If 25 or more equivalents of acetic anhydride were used, the product was indeed the acetate (34) Figure 5. Increasing the temperature to 30°C (14 equivalents of acetic anhydride) still resulted in the anhydride (32) as the major product, but at 40°C the acetate (34) was formed along with (32) as a 2:1 mixture. Interestingly, heating the diacid (31) to 100°C with only 1.2 equivalents of acetic anhydride again gave anhydride (32) as the sole product.

The differences between these results and those of Lewer and MacMillan can be rationalised if differences in reagent concentration and reaction temperatures are taken into account. In their synthetic procedure a large excess of acetic anhydride (*ca* 45 equivalents) was used. Their n.m.r. time course study used *ca* 27 equivalents of acetic anhydride and there was no indication of the n.m.r. probe temperature. In addition they stated that the desired product (32) was never formed exclusively and occurred for only a short period. However on comparing the n.m.r. spectra in their time course study with those in Figure 2 it appears that the signals at *ca* 2.75 and 2.9 p.p.m. which they have assigned to the methylene hydrogens of anhydride (32) and diacid (31) respectively should have their assignment reversed. When this is done it becomes apparent that the anhydride (32) is produced in significant quantities and for a significant time in their time course study before being eventually converted to the acetate (34).

Although the use of acetic anhydride is entirely satisfactory for the production on anhydride (32), it was found that an alternative

method not subject to the same variability was to react the diacid (31) with acetyl chloride and diethylamine in T.H.F. at $0^{o}C$. Reaction was again quantitative and was complete in only 2 h. As was described above the acetate (34) can be more conveniently prepared from the diacid (31) by heating in acetyl chloride at $50^{o}C$ for 4 hr. Removal of excess acetyl chloride gave a quantitative yield of (34). Anhyride (32) can also be converted readily to the corresponding acetate (34) by stirring at room temperature with a slight excess of acetyl chloride. Treatment of the anhydride (32) with sodium borohydride in propan-2-ol, acidification and continuous extraction with ether gave almost pure mevalonic acid lactone (33) which was purified finally by column chromatography on silica using hexane-ether as eluent.

3.6 EXPERIMENTAL

For general experimental conditions, see 2.8.1

Isodehydroacetic ester (17)

Ethyl acetoacetate (130g; 1 mol) was added (dropwise) to cooled (ice bath) concentrated sulphuric acid with stirring in the absence of moisture and allowed to stand at room temperature for six days. The thick yellow reaction mixture was then poured onto ice (500g) and diluted to one litre with water. This solution was extracted with ether (3 x 100ml). The ethereal solution was washed with sodium carbonate solution (3 x 50ml), water (2 x 70ml) and dried. The solvent was removed and the oily residue (57g) distilled (water pump pressure) to furnish isodehydroacetic ester (17) (43g; 43%).

b.p. 168-170^{o}C

δ_H 1.36 (3H, t, J 6Hz, CH_2 $C\underline{H}_3$); 2.21 (3H, d, J 2Hz, $C\underline{H}_3C=$); 2.37 (2H, s, $=CC\underline{H}_2O$); 4.34 (2H, q, J 6Hz, CH_3 $C\underline{H}_2O$); 5.99 (1H, s, $=C\underline{H}$).

3-Methylglutaconic acid (18)

Isodehydroacetic ester (17) (40g; 0.2mol) was dissolved in a 28% solution of potassium hydroxide (220ml). The mixture was heated under reflux with the flask inclined at an angle of 40^{o} to prevent loss during the violent evolution of alcohol vapour which occurred ten minutes after heating was started. Boiling was continued for 60 minutes. The mixture was then cooled to room temperature and cautiously acidified (6N H_2SO_4). The acid solution together with precipitated potassium sulphate was repeatedly extracted with ether (5 x 150ml). The dried ethereal solution gave, on evaporation, a semi solid residue which was freed from alcohol and acetic acid in an evacuated desiccator containing potassium hydroxide. Recrystallisation from chloroform gave 3-methylglutaconic acid (18) as a mixture of cis and trans isomers (23g; 80%) M.P. 110-113^{o}C.

4-Methyl-4-hydroxy-hepta-1,6-diene (30)

A solution of ethyl acetate (5.98g; 0.068 mol) and allyl bromide (25.5g; 0.204 mol) in a mixture of dry ether and T.H.F. (3/7; V/V) (100ml) was added to ice cold magnesium turnings (6.49g; 0.27 mol) in a mixture of dry ether and T.H.F. (20ml) at such a rate that a moderate reflux was maintained. After the addition was complete the reaction mixture was refluxed for one hour and stirred at room temperature for five hours. Ice (100g) was added to the mixture and it was cooled (ice bath) before being acidified with 6N H_2SO_4 (150ml). The acid solution was stirred overnight. The acidic mixture was then extracted with diethyl ether (3 x 100ml) and the combined ethereal solutions washed successively with saturated sodium hydrogen carbonate solution, water, sodium chloride solution and dried (Na_2SO_4). Removal of ether (in vacuo) left the crude product (7.5g) which distilled, at 82-85^{o} (water pump pressure). The yield of pure

4-methyl-4-hydroxyhepta-1,6-diene (30) was (4.6g; 95%).

δ_H 1.13 (3H, s, C$\underline{H}$); 2.21 (4H, d, J 7Hz, C$\underline{H}_2$); 2.52 (1H, s, exchangeable, O$\underline{H}$), 5.04-5.14 (4H, m, =C$\underline{H}_2$); 5.90 (2H, m,=C$\underline{H}$).

3-Methyl-3-hydroxypentan-1,5-dioic acid (31)

A solution of 4-methyl-4-hydroxy-hepta-1,6-diene (39) (1.31g; 10.39 mmol) in a 10:1 mixture of methylene chloride and acetic acid (20ml) was subjected to ozonolysis at -78°C (dry ice-acetone) for 1.5 hours. The sky blue solution was left overnight at room temperature. Methylene chloride was then evaporated (in vacuo) at 30°C. Acetic acid (10ml) and hydrogen peroxide (4ml) was added to the crude product and the mixture was heated under reflux for 24 hours. The yellow solution was cooled to room temperature and the solvent evaporated (in vacuo). The semi solid residue that solidifies on standing was recrystalised from ether to furnish 3-methyl-3-hydroxypentan-1,5-dioic acid (31) (1.5g; 96%).

m.p. 108-110°C (lit m.p. 110-111°C).[21] (Found C, 44.58; H, 6.30, $C_6H_{10}O_5$ requires C, 44.44; H, 6.17%)

δ_H (d_6-acetone) 1.41 (3H, s, C$\underline{H}_3$); 2.7 (4H, s, C$\underline{H}_2$); 8.54 (3H, s, exchangeable, O$\underline{H}$).

ν_{max} (KBr) 3025(Broad), 1720cm^{-1}.

3-Hydroxy-3-methylpentane-1,5-dioic anhydride (32)

A mixture of diacid (31) (810mg; 5 mmol) and acetic anhydride (6.6ml, 70mmol) was stirred at room temperature. After 72 hours the initial suspension had turned into a clear solution. The excess of acetic anhydride was removed under high vacuum to give a white solid (817mg) which was recrystallised from diethyl ether-light petroleum (b.p. 30-40°C) to give 3-hydroxy-3-methylpentane-1,5-dioic anhydride (32) as needles (673 mg), m.p. 102-103°C (lit.,101-102.5°C);[22] ν_{max} 3320, 1820, 1768 and 1755 cm^{-1}; δ_H (d_6-acetone) (200 MHz) 1.44 (3H,

s, $C\underline{H}_3$); 2.80, 2.90, 2.98 and 3.06 (4H, AA'BB', $C\underline{H}_2$) and 4.67(1H, brs, exchangeable, O<u>H</u>) (Found C, 50.1; H, 5.45 C_6 H_8 O_4 requires, C, 50.00; H, 5.56%).

Mevalonic acid lactone (33)

The crude anhydride (32) (0.75g) was dissolved in propan-2-ol (20ml) and the solution was added dropwise to sodium borohydride (0.4g) cooled in an ice-bath. The reaction mixture was stirred overnight at room temperature. After removal of the solvent, water (10ml) was added and the mixture was acidified to pH2 in an ice-bath. the solution was extracted continuously with diethyl ether for 45 hours. The extract was dried over magnesium sulphate and the solvent removed on a rotary evaporator to give an oil which was shown by t.l.c. to have one component, corresponding to mevalonic acid lactone. Column chromatography on Malinckrodt silica AR-CC-7(20g) and elution with hexane-diethyl ether (2:8) gave pure mevalonic acid lactone (33) (300mg; 75.6%).

3-Acetoxy-3-methylpentane-1,5-dioic acid anhydride (34)

3-Hydroxy-3-methylpentane-1,5-dioic acid (31) (1.55g; 6.56 mmol) was dissolved in acetyl chloride (4.5g; 57.3 mmol) and the mixture was heated under reflux at 60° for two hours. Removal of excess acetyl chloride (in vacuo) gave a semi solid residue that was freed from acetic acid in an evacuated dessicator containing potassium hydroxide to give (34) (1.77g; 100%) which was recrystallised from ether-light petroleum (30-40°C) to give colourless needles.

m.p. 83-85°C(lit m.p. 85°C).[23] (Found: C,51.68; H, 5.48. C_8 H_{10} O_5 requires C, 51.60; H, 5.37%). ν max 1810, 1775, 1760 and 1735cm^{-1}.

δ_H 1.68 (3H, s, $C\underline{H}_3$); 1.98 (3H, s, $C\underline{H}_3CO$); 3.28 (4H, AA' BB', multiplet, $C\underline{H}_2$).

4-Methylglutaconic anhydride (19)

A solution of 3-acetyl-3-methylpentane-1,5-dioic acid anhydride (34) (1.55g; 9.55 mmol) in xylene (25ml) was heated under reflux for 20 hours. The hot solution was filtered and the solvent removed (in vacuo) to give 4-methylglutaconic anhydride (19) in quantitative yield. Recrystallisation from ether-light petroleum (b.p.30-40^{o}) gave crystals, m.p. 87-88^{o}C. (Found C, 56.86; H, 4.86. $C_6H_6O_3$ requires C, 57.14; H, 4.70%).

6-Methoxy-4-methyl-pyran-2-one (20)

An ice cold solution of 4-methylglutaconic anhydride (1g; 7.93 mmol) in dry ether (5ml) was reacted with an ethereal solution of diazomthane with stirring. Stirring was continued for one hour at room temperature. The pale yellow solution was then filtered through celite and solvent evaporated (in vacuo) to give a thick oil that was recrystallised from ether-light petroleum (30-40^{o}) to furnish (20) (0.92g; 83%) as fine colourless needles, m.p. 54-55^{o}C.

(Found C, 59.78; H, 5.44. $C_7H_8O_3$ requires C, 60.00; H, 5.71%).

$C_7H_8O_3$=140, P^{+}=140.

ν_{max} ($CHCl_3$) 1735, 1675 and 1535cm^{-1}.

δ H 2.16 (3H, s, $C\underline{H}_3$); 3.87 (3H, s, $OC\underline{H}_3$); 5.30 (1H, s, =C$\underline{H}$); 5.71(1H, s, =C$\underline{H}$)

6-Silyloxy-4-methylpyran-2-one (59)

4-methylglutaconic anhydride (3.693g; 0.029mol) in dry benzene (70ml) was treated with bis(trimethylsilyl) acetamide at 30-35^{o}C for one hour. The benzene was removed by rotary evaporation and the liquid residue was distilled to furnish the highly moisture sensitive (59) (4.02g; 58%). b.p. 80-82 (2mm Hg).

δ H 0.15 (9H, s, $C\underline{H}_3$-Si); 1.85 (3H, s, $C\underline{H}_3$); 5.00 (1H, s, =C$\underline{H}$); 5.44 (1H, s, =C$\underline{H}$).

8-Hydroxy-1-methoxy-3-methylanthraquinone (28)

A solution of 6-methoxy-4-methyl-pyran-2-one (20) (20mg; 0.144 mmol) and juglone (50mg; 0.288 mmol) in xylene (2ml) was refluxed for five days, cooled and treated with silver oxide (125mg) and anhydrous magnesium sulphate with stirring at room temperature for 12 hours. The mixture was filtered, and the solvent removed. The solid residue then was subjected to preparative thick layer chromatography using methylene chloride as developing solvent. The band (Rf 0.43) was separated and eluted with chloroform to give 8-hydroxy-1-methoxy-3-methyl anthraquinone as a yellow solid (24mg; 62%). Recrystallisation from methanol gave yellow crystals, m.p. 204-205°C. (Found, C, 71.79; H, 4.27. $C_{18}H_{12}O_4$ requires C, 71.64; H, 4.47). δ H 2.38 (3H, s, CH_3); 3.84 (3H, s, OCH_3); 6.97 (2H, m, H-2 and H-3); 7.18 (1H, dd, J 8,2Hz, H-7); 7.50 (1H, t, J 8Hz, H-6); 7.60 (1H, dd, J 8,2Hz, H-5).

1,8-Dihydroxy-3-methylanthraquinone(Chrysophanol) (24)

A solution of 8-hydroxy-1-methoxy-3-methylanthraquinone (24mg; 0.089 mmol) and 48% hydrobromic acid (1.5ml) in glacial acetic acid (4ml) was refluxed for five hours. The cooled solution was diluted with water, extracted with dichloromethane, and the organic phase washed with water, dried over sodium sulphate, filtered and evaporated (in vacuo) to give (24) (23mg; 100%) which was recrystallised from methanol. m.p. 197-198°C. (Found C, 71.70; H, 4.27. $C_{15}H_{10}O_4$ requires C, 70.86; H, 3.93%).

3-Methyl-1,4,8-trihydroxyanthraquinone (Islandicin) (38)

(a) Chrysophanol (497mg; 1.95 mmol) was dissolved with shaking in a solution of boric acid (1.24g) in 65% oleum (80ml) in a sealed flask. The mixture was allowed to stand for 10 days at room temperature when the initially red solution had turned blue. The

solution was poured dropwise onto ice (700g) and the orange precipitate collected by centrifugation dissolved in 0.2M potassium hydroxide (1.5l) under nitrogen and allowed to stand at room temperature for 15 minutes. the solution was acidified with hydrochloric acid (6N) and heated with butanol (200ml) on a boiling water bath for one hour. The orange-red extract was washed with water and solvent evaporated under vacuum. The residue was subjected to preparative t.l.c. in chloroform/toluene (1:3) and the major orange band (Rf 0.7) eluted to give islandicin (38) (287mg; 56%) which was recrystallised from ethyl acetate to give dark red prisms. m.p. 222°C (lit m.p. 218°C).

(b) The following modified procedure gave an improved yield. Chrysophanol (24) (98mg; 0.38 mmol) was dissolved in a solution of boric acid (254mg) in 65% oleum (15ml) in a sealed flask and the hydroxylation was carried out as before. After the solvent (butanol) had been removed acetic anhydride (10ml) together with sulphuric acid (three drops) was added to the deep red residue and the reaction mixture was stirred at room temperature for one hour. The pale yellow mixture was then poured into water (100ml) and extracted with dichloromethane (3 x 15ml). The solvent was evaporated (in vacuo) and the yellow residue was purified by t.l.c. using methylene chloride as developing solvent. The band (Rf0.31) was eluted with chloroform to give islandicin triacetate (100mg) which was recrystallised from light petroleum (60-80°) to give pale yellow needles. m.p. 192-193°C. δ_H 2.29 (3H, s, $C\underline{H}_3$); 2.28 (6H, s, $C\underline{H}_3$); 2.46 (3H, s, $C\underline{H}_3$); 7.24 (1H, d, J 1Hz, H-2); 7.29(1H, dd, J 8, 2Hz, H-7); 7.67(1H, t, J 8Hz, H-6); 8.05(1H, dd, J 8,2Hz,H-5).

The above product was then dissolved in aqueous methanol (10ml);

to it was added concentrated hydrochloric acid (10 drops) and the solution was refluxed for 15 hours. The red precipitate was filtered washed with water (20 drops) and dried to furnish pure islandicin (38) (62mg; 60%).

Hydroxylation of 1,5-dihydroxy-3-methylanthraquinone

1,5-Dihydroxy-3-methylanthraquinone (ziganein) (50mg; 0.19 mmol) was dissolved in a solution of boric acid (150mg) in 65% oleum (10ml) in a sealed flask. The mixture was allowed to stand at room temperature for 60 days with occasional shaking. Work up procedure was followed as previously described. After the removal of the solvent (butanol), acetic anhydride (5ml) and sulphuric acid (1 drop) was added to the deep orange residue and stirred at room temperature for one hour. The usual work up procedure was followed as described before and the residue was subjected to preparative t.l.c. in methylene chloride to give one major band (Rf 0.25) that was found to be 1,5-acetoxy-3-acetoxymethylanthraquinone (41) (25mg; 32.05%) which was recrystallised from methanol to yield pale yellow crystals as needles m.p. 181-182°C.

δ_H, 2.16 (3H, s, $C\underline{H}_3CO$); 2.45 (6H, s, Ar-$C\underline{H}_3$ CO); 5.19 (2H, s, $C\underline{H}_2$-$OOCH_3$); 7.36 (1H, d, J 2Hz, H-2); 7.40 (1H, dd, J 8,2Hz, H-6); 7.84(1H, t, J 8Hz, H-7); 8.16 (1H, d, J 2Hz, H-4); 4.20 (1H, dd, J 8,2Hz, H-8).

Bromoacetaldehyde Dimethyl Acetal (51)

Vinyl acetate (43g; 0.5mol) was added to carbon tetrachloride (75ml) and cooled in an ice water mixture. A mixture of carbon tetrachloride (45ml) and bromine (80g; 0.5mol) was added to the vinyl acetate mixture with shaking, care being taken not to allow the temperature to rise above 10°. The addition took place about 15 minutes and the end point was reached when bromine no longer

decolourised. The brominated mixture was then added to 175ml of methanol (99.5-100%) and cooled during the first 30 minutes to prevent rise in temperature. It was shaken occasionally and allowed to stand for two days. The mixture which consisted of two layers was diluted with one litre of water and the bromoacetal-carbon tetrachloride layer drawnoff. The methyl alcohol water mixture was further extracted with (2 x 75ml) of carbon tetrachloride. The carbon tetrachloride solutions were combined and distilled through a Vigreux type column, to give pure bromoacetaldehyde dimethyl acetal (51) (65g; 77%).

1,1-Dimethoxyethene (49)

Bromoacetaldehyde dimethyl acetal (65g; 0.38 mol) was added to a solution of potassium metal (15g; 0.38mol) in dry ∝-terpineol (450ml). The mixture was allowed to reflux under nitrogen for five minutes at 120-200°C and distilled on a Vigreux type column to give a reasonably pure product (49), (25g).

5,7-Dimethoxy-1,4-naphthoquinone (47)

1,4-benzoquinone (48) (100mg; 0.92 mmol) and 1,1-dimethoxyethene (750mg; 8.52 mmol) were allowed to stand in dimethyl sulfoxide for 24 hours. Chromatography t.l.c. in benzene/ethyl acetate (10:1) and rechromatography in chloroform gave one major yellow band which was eluted with chloroform to give a bright yellow solid (95mg; 47%). Recrystallisation from methanol gave yellow needles m.p. 164-166°C.

1,2-Dicarbomethoxy-3-methoxy-5-methyl benzene (60)

A solution of 6-methoxy-4-methyl-pyran-2-one (20) (140mg; 1 mmol) and dimethyl acetylenedicarboxylate (248mg; 2 mmol) in dry toluene (6ml) was refluxed under dry atmosphere for 18 hours. The solvent was evaporated (in vacuo) and the residue was subjected to t.l.c. in ethyl acetate/n-hexane (1:3; V/V). The band (Rf0.4) was isolated and eluted with chloroform to give (60) (200mg; 84%) which

was recrystallised from ether-light petroleum (30-40°) to yield colourless prisms. m.p.87.5-88.5°C. $C_{12}H_{14}O_5$ = 238; p^+238. ν max (KBr) 1740, 1720cm^{-1}.

δH 2.34 (3H, s, CH_3); 3.78 (3H, s, OCH_3); 3.81 (3H, s, OCH_3); 3.86 (3H, s, OCH_3); 6.90(1H, d, J 2Hz, H-4); 7.36(1H, d, J 2Hz, H-6).

Maleic anhydride-methoxypyranone adduct (66)

A solution of 6-methoxy-4-methyl-pyran-2-one (20) (140mg; 1 mmol) and maleic anhydride (140mg; 1.42 m mol) in dry xylene was heated under reflux for 48 hours. The reaction mixture was cooled to room temperature and the crystalline precipitate was filtered washed with a few drops of toluene and dried to furnish the adduct (66, 169mg) which was recrystallised from benzene to yield colourless leaflets. m.p. 273-274°C.

$C_{14}H_{12}O_7$ = 292; P^+: 292. νmax (KBr) 1860, 1800, 1200, 1095, 940cm^{-1}.

δH 1.78 (3H, d, J 2Hz, CH_3); 3.45 (1H, t of d, J 3, 2Hz, H-a); 3.65 (3H, s, OCH_3); 3.86 (1H, dd, J 9, 2Hz, H-d); 4.10 (1H, d, J 9Hz, H-C); 5.84 (1H, dd, J 2,2Hz, H-b).

5-Methoxy-7-methyl-naphthoquinone-1,4 (65)

A toluene solution of 6-methoxy-4-methyl-pyran-2-one (20) (140mg; 1 mmol) and 1,4-benzoquinone (64) (216mg; 2 mmol) was refluxed for 14 hours. Cooled and treated with silver oxide (250mg) and magnesium sulphate (500mg) with stirring at room temperature for 12 hours. The mixture was filtered and the solvent removed (in vacuo). The residue was subjected to thin layer chromatography in methylene chloride. The major yellow band (Rf0.31) isolated and eluted to give a yellow product (86mg) which was recrystallised from ethanol to give bright yellow needles. m.p. 173.5-174.5°C. $C_{12}H_{10}O_3$ =202; p^+:202.

νmax(KBr) 1664, 1650, 1600cm^{-1}. δH 2.45 (3H, s, CH_3); 3.97 (3H, s, OCH_3); 6.80 (2H, s, H-2 and H-3); 7.10 (1H, d, J 2Hz, H-6); 7.63 (1H, s, J 2Hz, H-8).

1-Carbomethoxy-2-methoxy-4-methyl benzene (62)

A solution of 6-methoxy-4-methyl-pyran-2-one (20) (140mg; 1 mmol) and methyl propiolate in dry xylene (6ml) was refluxed for five days. the solvent was removed (in vacuo) and the residue fractionated by t.l.c. using 20% ethyl acetate in n-hexane as eluent. Two bands were eluted the band (Rf0.3) was the expected product (62) (37mg; 20%) and the band Rf0.59 appeared to be 1-carbomethoxy 2-hydroxy-4-methyl benzene (63) (10mg; 6%) both pale yellow oils. ν_{max} ($CHCl_3$) 1725, 1615, 1295, 1090cm^{-1}. δH 2.34 (3H, s, CH_3); 3.82 (3H, s, OCH_3); 3.84 (3H, s, CO_2CH_3); 6.76 (2H, m, H-5 and H-3); 7.69 (1H, d, J 8Hz, H-6).

1,2-Dicarboxmethoxy-3-hydroxy-5-methyl benzene (61)

A solution of 6-silyloxy-4-methyl-pyran-2-one (20) (249mg; 1.25 mmol) and dimethyl acetylene dicarboxylate (357mg; 2 mmol) in dry benzene (10ml) was heated under reflux in a dry atmosphere for five days. The solvent was evaporated (in vacuo) and the residue was dissolved in methanol 95% (10ml). The methanolic solution was stirred for five hours. The solvent was removed and the residue purified by t.l.c. using 20% ethyl acetate in n-hexane as developing solvent. The band (Rf0.37) was isolated and eluted with chloroform to give (61) which was recrystallised from ether-light petroleum (30-40^o) to yield colourless needles (162mg; 72%). m.p. 75-76^{o}C .
$C_{11}H_{12}O_5$ = 224; P^+: 224. ν max (KBr) 1780, 1690, 1620cm^{-1}. δH 2.32 (3H, s, CH_3); 3.84 (3H, s, OCH_3); 3.87 (3H, s, CH_3); 6.76(1H, d, J 2Hz, H-6); 6.86(1H, d, J 2Hz, H-8); 10.60(1H, brs, OH, exchangeable).

1-Carbomethoxy-2-hydroxy-4-methyl benzene (63)

A solution of 6-silyloxy-4-methoxy-pyran-2-one (59) (341mg; 1.72 mmol) and methyl propiolate (144mg; 1.72 mmol) in dry xylene was

refluxed (in a dry atmosphere) for five days. The solvent was removed and wet methyl alcohol was added to the residue. The mixture was stirred at room temperature for 4 hours. Evaporation of the solvent gave pale yellow oil which was purified by t.l.c. using 10% ethyl acetate in n-hexane as developing solvent. The major band (Rf0.62) was separated and eluted with chloroform to furnish an oil which was found to be (63) (186mg; 65.2%).

$C_9H_{10}O_3$ = 166; p^+: 166. νmax. 3500, 1715, 1615, 1290, 1090cm^{-1}.

δH 2.32 (3H, s, $C\underline{H}_3$); 3.88(3H, s, CO_2 $C\underline{H}_3$); 6.67 (1H, dd, J 8,2Hz, H-5); 6.78 (1H, d, J 2Hz, H-3); 7.68 (1H, d, J 8Hz, H-6); 10.69 (1H, s, O$\underline{H}$, exchangeable).

4-Hydroxy-4-pentyl-hepta-1,6-diene (74)

Ethyl hexanoate (73) (10g) and allyl bromide (18ml;3eq) were dissolved in a mixture of ether and T.H.F. (3/1; v/V) (100ml). The solution was added slowly to magnesium turnings (6.6g;4eq) in ether-T.H.F. (1/1, V/V) (20ml). After the reaction was complete (18 hours) the reaction mixture was worked up as for (39) to yield the expected diene (76%) which was purified by distillation b.p. 106^{o}C.

δH 0.89 (3H, t, J 6Hz, $C\underline{H}_3$); 1.20-1.55 (8H, m, $C\underline{H}_2$); 1.69(1H, s, O$\underline{H}$, exchangeable); 2.2 (4H, d, J 8Hz, $C\underline{H}_2$); 4.95-5.15 (4H, m, $C\underline{H}_2$ = C); 5.6-6.1(2H, m, C = C$\underline{H}$).

3-Hydroxy-3-pentyl-pentanedioic acid (75)

4-Hydroxy-4-pentyl-hepta-1,6-diene (5g) was dissolved in a mixture of methylene chloride and glacial acetic acid (5/1; V/V) (55ml) and ozonolysis was carried out as for (39). Oxidation of the ozonide followed by evaporation of the solvents gave a pale yellow residue which solidified on standing. Recrystallisation from ether-xylene gave (75) as colourless crystals. m.p. 89.5-91.0^{o}C.

$C_{10}H_{18}O_5$ = 218; P^+; 219. (Found C, 55.0; H, 8.17. $C_{10}H_{18}O_5$ requires C, 55.04; H, 8.25%). δH (d6-acetone) 0.76 (3H, t, J 5Hz,

$C\underline{H}_3$); 1.0-1.7 (8H, m. CH_2); 2.60 (4H, s, $C\underline{H}_2$); 7.03 (2H, brs, COO$\underline{H}$, exchangeable).

4-Pentylglutaconic anhydride

Acetyl chloride (3 eq) was added to 3-hydroxy-3-pentyl-pentanedioic acid (4g) and the mixture was refluxed for two hours. The excess acetyl chloride from the above reaction mixture was removed (in vacuo) and the residue was dissolved in xylene (70ml). The solution was refluxed for 24 hours. Removal of the solvent gave 4-pentyl-glutaconic anhydride which was used unpurified for the next step.

δ_H, 0.90 (3H, t, J 5Hz, $C\underline{H}_3$); 1.1-1.7 (8H, m, $C\underline{H}_2$) 3.43 (2H, s, $C\underline{H}_2$); 6.00 (1H, s, = C$\underline{H}$).

6-Methoxy-4-pentyl-pyran-2-one (76)

The above anhydride was reacted with diazomethane (excess) to yield (76) which was recrystallised from ether-light petroleum (30-40^o) to give colourless crystals m.p. 28-30^{o}C. (Found C, 67.57; H, 8.21. $C_{11}H_{16}O_3$ requires C, 67.34; H, 8.16%). δ H 0.86 (3H, t, J 5Hz, $C\underline{H}_3$); 1.0-1.5 (8H, m, $C\underline{H}_2$); 3.87 (3H, s, $OC\underline{H}_3$); 5.23 (1H, s, H-5); 5.62 (1H, s, H-3).

1,2-Dicarbomethoxy-3-methoxy-5-pentyl benzene (77)

The 6-methoxy-4-pentyl-pyran-2-one (76) (0.364g) was dissolved in dry toluene (30ml) and dimethyl acetylene dicarboxylate (4 eq; 1.092g) was added to this solution. The mixture was refluxed for 24 hours. Removal of the solvent gave a crude product which was purified by t.l.c. using 20% ethyl acetate in light petroleum (40-60^o) as eluent to give (77) as an oil (0.47g; 87%). δ H 0.89 (3H, t, J 6Hz, $C\underline{H}_3$); 1.2-1.4 (8H, m, CH_2); 3.80 (3H, s, $OC\underline{H}_3$); 3.84 (3H, s, CO_2 $C\underline{H}_3$); 3.89 (3H, s, CO_2 $C\underline{H}_3$); 6.94 (1H, d, J: Hz, H-4); 7.40 (1H, d, J 1Hz, H-6).

4-Hydroxy-4-phenyl-hept-1,6-diene (79)

Ethyl benzoate (78) (10g), together with allyl bromide (16ml) and magnesium turnings (6.6g) were reactd together as before to yield the corresponding diene (9.37g; 74%)

δ_H 2.22 (1H, s, OH, exchangeable); 2.58 (4H, d, CH_2); 4.9-5.2 (4H, m, CH_2); 5.4-5.9 (2H, m, C=CH); 7.1-7.5 (5H, m, Ar-H).

3-Hydroxy-3-phenyl-pentanedioic acid (81)

Ozonolysis of the diene and oxidation of the ozonide was carried out as before to give the acid (81) which was recrystallised from benzene to give pure crystals. m.p. 141-5.1-144°C. (Found C,59.1; H,5.3 $C_{11}H_{12}O_5$ requires C, 55.9; H, 5.4%). δ_H (d6-acetone) 3.05 (4H, s, CH_2); 7.1-7.6 (5H, m, ArH); 8.2-9.0(2H, brs, OH, exchangeable).

4-Acetyl-4-phenyl-glutaconic anhydride

Acetyl chloride (5ml) was added to the acid (3.54g) and the mixture was refluxed for six hours. Removal of the solvent gave a solid which was recrystallised from benzene to give colourless crystals. m.p. 127-192°C. (Found C, 63.0; H, 4.6 $C_{13}H_{12}O_5$; requires C, 62.9; H, 4.8%). Found M^+248, $C_{13}H_{12}O_5$ requires M, 248. ν_{max} ($CHCl_3$) 1880, 1780, 1600, 1440 cm^{-1}. δ_H 2.00 (3H, s, COCH); 3.88-3.14 (4H, d, J 16Hz, CH_2); 7.2-7.5 (5H, m, ArH).

4-Phenyl-glutaconic anhydride

4-acetyl-4-phenyl-glutaconic anhydride was pyrolysed as for the pentyl analogue. Removal of the solvent gave a solid which was recrystallised from benzene to yield colourless crystals. m.p. 175-185°C(decom). δ_H (d_6-DMSO) 2.97 (2H, s, CH_2); 6.10 (1H, s, C=CH); 6.7-7.2 (5H, m, ArH). (Found C, 70.0; H,4.4 $C_{11}H_8O_3$ requires C, 70.2; H, 4.2%).

6-Methoxy-4-phenyl-pyran-2-one (82)

4-Phenyl-glutaconic anhydride was methylated as for the pentyl

analogue to give a crystalline product. (Found C, 71.4; H,5.0 $C_{12}H_{10}O_3$ requires C, 71.3; H, 5.0%). δ_H 3.94 (3H, 3 OCH_3); 5.64 (1H, d, J 1Hz, CH); 6.04 (1H, d, J 1Hz, CH); 7.30-7.60 (5H, m, ArH).

1,2-Dicarbomethoxy-3-methoxy-5-phenyl benzene (83)

The 6-methoxy-4-phenyl-pyran-2-one (82) and dimethyl acetylene dicarboxylate (4 eq) were dissolved in dry toluene (30ml). The mixture was refluxed for 72 hours. The solvent was removed and the oily residue was purified by t.l.c. using 15% ethyl acetate in light petroleum (40-60°) as developing solvent. the only major band was removed and eluted with chloroform to give a solid which was recrystallised from ether-light petroleum (30-40°) to yield colourless needles (83) (95%) m.p. 142.5-144.0°C. (Found C, 68.04; H, 5.20. $C_{17}H_{16}O_5$ requires C, 68.00; H, 5.33%). δ_H 3.90 (3H, s, OCH_3; 3.92 (3H, s, CO_2 CH_3; 3.97 (3H, s, CO_2 CH_3); 7.30 (1H, s, C=CH); 7.38-7.58 (5H, m, ArH); 7.85 (1H, s, C=CH).

4-Hydroxy-3,4,5-trimethyl-hepta-1,6-diene (83)

Grignard reaction of ethyl acetate (6g), 3-chloro-1-butene (18.5g) and magnesium turnings (6.5g) followed by the usual work up gave the corresponding diene (8.6g; 82%) which distilled at 68-71°C (water pump pressure) to yield (83) as colourless liquid.

δ_H 1.01 (3H, s, CH_3); 1.02 (3H, s, CH_3); 1.05 (3H, s, CH_3); 1.41 (1H, s, OH, exchangeable), 2.20-2.55 (2H, m, C-CH); 4.90-5.20 (4H, m, $C=CH_2$); 5.6-6.1(2H, m, C=CH).

3-Hydroxy-2,3,4-trimethyl pentanedioic acid (84)

Ozonolysis of the diene (83) (4.4g) was carried out as for the pentyl analogue (four hours). After the usual work up a thick residue was obtained which was used unpurified for the next step.

δ_H(D_6-acetone) 0.60 (3H, s, CH_3); 0.68 (3H, s, CH_3) 9.73 (3H, s, CH_3); 1.9-2.4 (2H, m, C-CH); 5.40 (2H, s, COOH, exchangeable).

4-Acetyl-3,4,5-trimethyl glutaric anhydride (85)

The cyclisation of the anhydride (0.56g) was carried out by the same method as for the pentyl derivative to yield the corresponding anhydride (0.58g; 93%) which was used without further purification.

3,4,5-Trimethyl glutaconic anhydride (86)

The pyrolysis was carried out in the same way as previously to yield a pale yellow gum (0.35g; 82%).

6-Methoxy-3,4,5-trimethyl-pyran-2-one (87)

Methylation of the above anhydride was carried out as before to give (88; 96%) which was used unpurified in the Diels-Alder reaction.

1,2-Dicarbomethoxy-3-methoxy-4,5,6-trimethyl benzene (88)

The 6-methoxy-3,4,5-trimethyl- pyran-2-one (87) (0.188g) and dimethyl acetylene dicarboxylate (0.49g; 4 equiv.) in dry toluene (10ml) was refluxed for 72 hours. Removal of the solvent gave a residue which was purified by t.l.c. using 17% ethyl acetate in light petroleum (40-60^{o}) as developing solvent. the only major band was removed and eluted with chloroform to give (88) as a colourless solid. Recrystallisation yielded colourless needles (0.06g; 38%) m.p. 72-73^{o}C. (Found C, 63.09; H, 6.58. $C_{14}H_{18}O_5$, requires C, 63.15; H, 6.76). δ H 1.2 (3H, s, $OC\underline{H}_3$); 1.48 (3H, s, $C\underline{H}_3$); 2.23 (3H, s, $C\underline{H}_3$); 2.48 (3H, s, $OC\underline{H}_3$); 3.95(3H, s, $CO_2\ C\underline{H}_3$); 4.07(3H, s, $CO_2\ C\underline{H}_3$).

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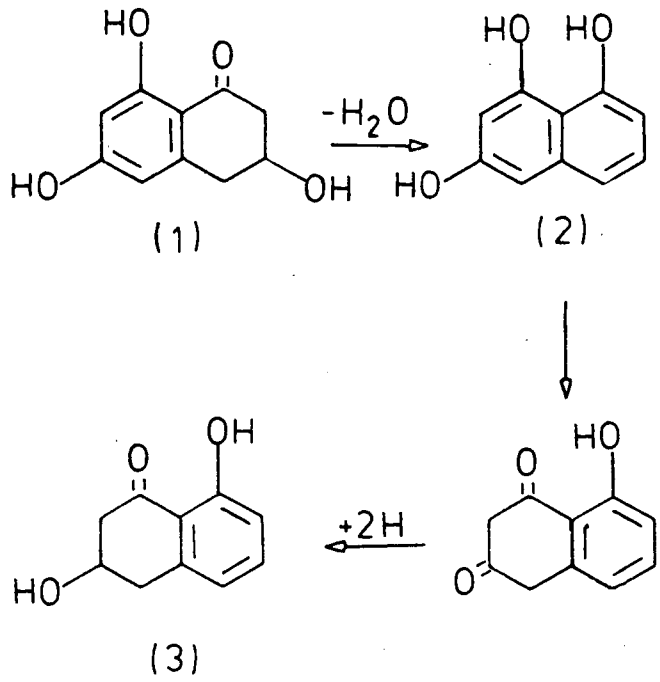
CHAPTER 4

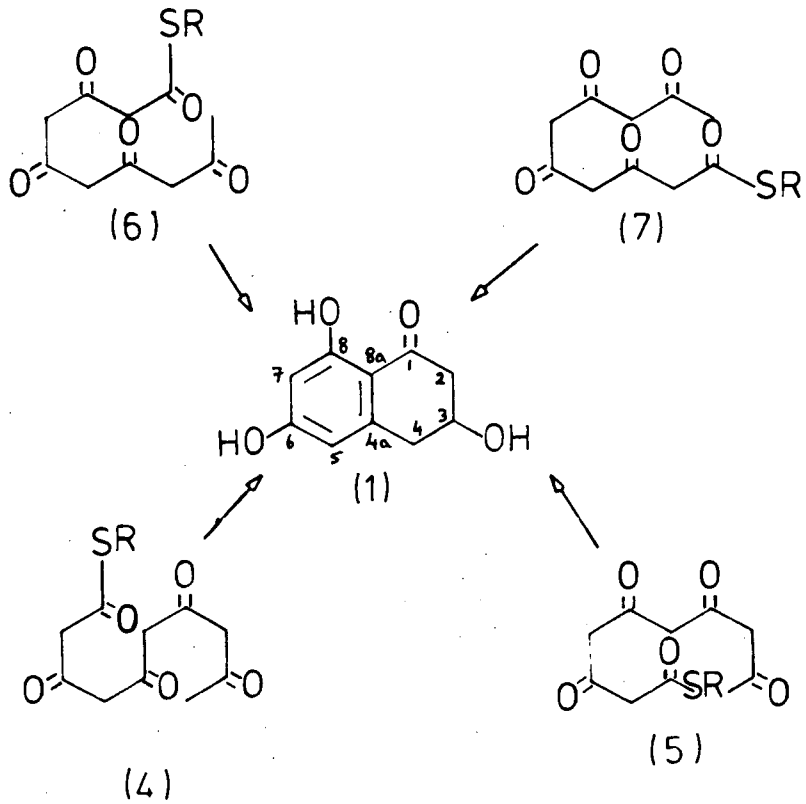
BIOSYNTHETIC STUDIES ON SCYTALONE

TABLE 1 ^{13}C chemical shifts for scytalone (1)

Carbon	Sc(p.p.m.)
1	202.1
8	166.0
6	165.4
4a	145.8
8a	111.5
5	108.9
7	101.3
3	66.4
2	47.1
4	38.9



Scheme 1



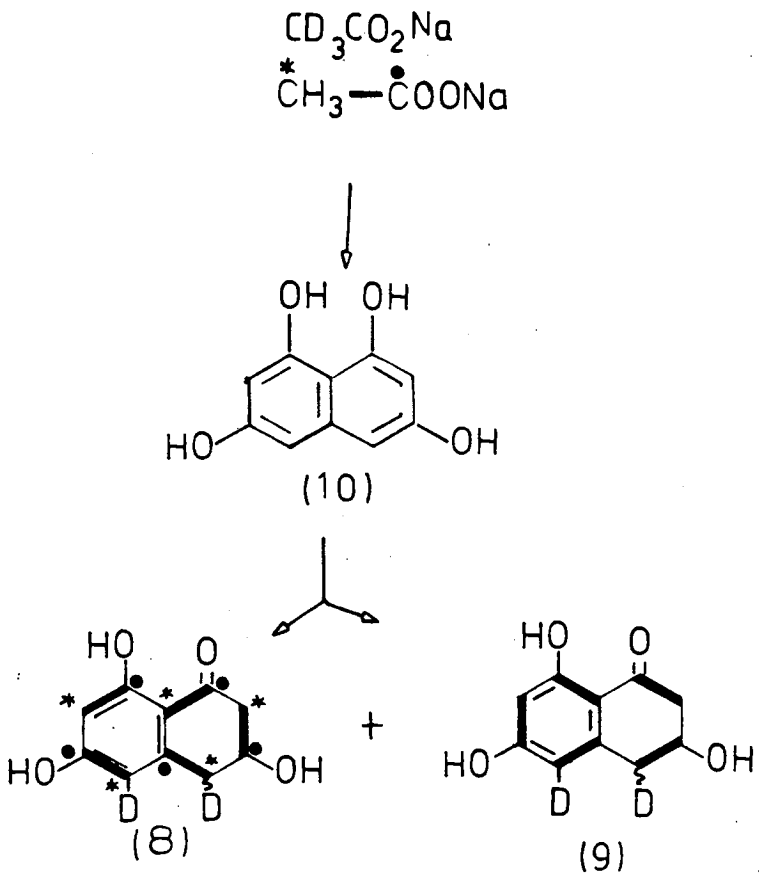
Scheme 2

4. BIOSYNTHETIC STUDIES ON SCYTALONE

4.1 INTRODUCTION

Scytalone [3,4-dihydro-3,6,8-trihydroxynaphthalene-2(1H)-one] (1) is a fungal metabolite which was first isolated from a species of Scytalidium.[1] It has also been isolated from Phialophora lagerbergii,[2] and Verticillum dahliae[3] in which it has been shown to be an intermediate on the pathway to fungal melanin.[4] Knowing that a melanin deficient mutant (brm-1) of Verticillum dahliae accumulated (+)-scytalone which when fed to a different mutant (brm-2) was enzymatically dehydrated to 1,3,8-trihydroxynaphthalene (2), Stipanovic et al fed 1,3,8-trihydroxy naphthalene to the brm-1 mutant to determine if it would be converted to melanin. No melanin was formed but a new compound (-)-vermelone [3,4 dihydro-3,8-dihydroxy-1(2H)-naphthalenone](3) was produced in quantity which in turn was converted to black melanin by the (brm-2) mutant. Therefore, (+)-scytalone appears to be on the biosynthetic pathway leading to fungal melanin. It appears that, scytalone dehydrates to give 1,3,8-trihydroxynaphthalene which in turn is reduced to (-) vermelone as summarised in Scheme 1.

The oxygenation pattern of scytalone strongly suggested a pentaketide origin. A number of alternative foldings of a pentaketide chain are consistent with the observed oxygenation pattern and these are summarised in Scheme 2. The ^{13}C n.m.r. spectrum of scytalone showed ten well resolved signals which have been assigned [2,5,6] as shown in Table 1. In the spectrum of scytalone produced by P. lagerbergii grown in the presence of sodium [1-^{13}C]acetate the signals due to C-1, C-3, C-4a, C-6 and C-8 were enhanced to twice their natural intensity thus confirming the pentaketide origin of scytalone.[2] Sankawa and co-workers[5] also incorporated [1,2-$^{13}C_2$]



Scheme 3

-acetate into scytalone using P.lagerbergii and in the ^{13}C n.m.r. spectrum of the labelled scytalone, eight of the ten signals showed multiple splitting patterns. For each ^{13}C natural abundance peak four satellite peaks were observed. This phenomenon was attributed to two ^{13}C - ^{13}C couplings arising as the result of incorporation of intact acetate units into scytalone with two different dispositions, i.e. two different arrangements of acetate units (8) and (9) (Scheme 3). Since no ^{13}C - ^{13}C coupling was observed between C-4a and C8a the participation of the pentaketide folding pattern (4) in Scheme 2 was ruled out.

The same group also incorporated [2-^{13}C, $^{2}H_3$]acetate. The ^{13}C-n.m.r. spectrum of the enriched scytalone showed enhancements for C-2, C-4, C-5, C-7 and C-8a indicating extensive loss of ^{2}H in the biosynthetic process. Only C-4 showed ^{13}C - H coupling, but since the corresponding signal was a triplet (1:1:1, J=20 Hz) it was concluded that this carbon (C-4) carries just one ^{2}H atom. Judging from its lower signal intensity, C-5 also seemed to be labelled by ^{2}H to a significant extent, but no ^{13}C - ^{2}H coupled signals could be observed. The fact that no deuterium was present at C-2 and C-7 was interpreted as there being little or no incorporation of ^{2}H at these two positions compared with C-4 and C-5.

As a result of these experiments it was suggested that a symmetrical intermediate such as 1,3,6,8-tetrahydroxynaphthalene (10) was involved in the biosynthesis of scytalone. Indeed it has been shown that $NaBH_4$ reduction of 1,3,6,8-tetrahydroxynaphthalene (10) gives racemic scytalone.[7] Thus the presence of two different arrangements (8) and (9) could be explained by assuming that the necessary reduction of the aromatic ring takes place in each of the rings of (10) with an equal probability. Similar results and

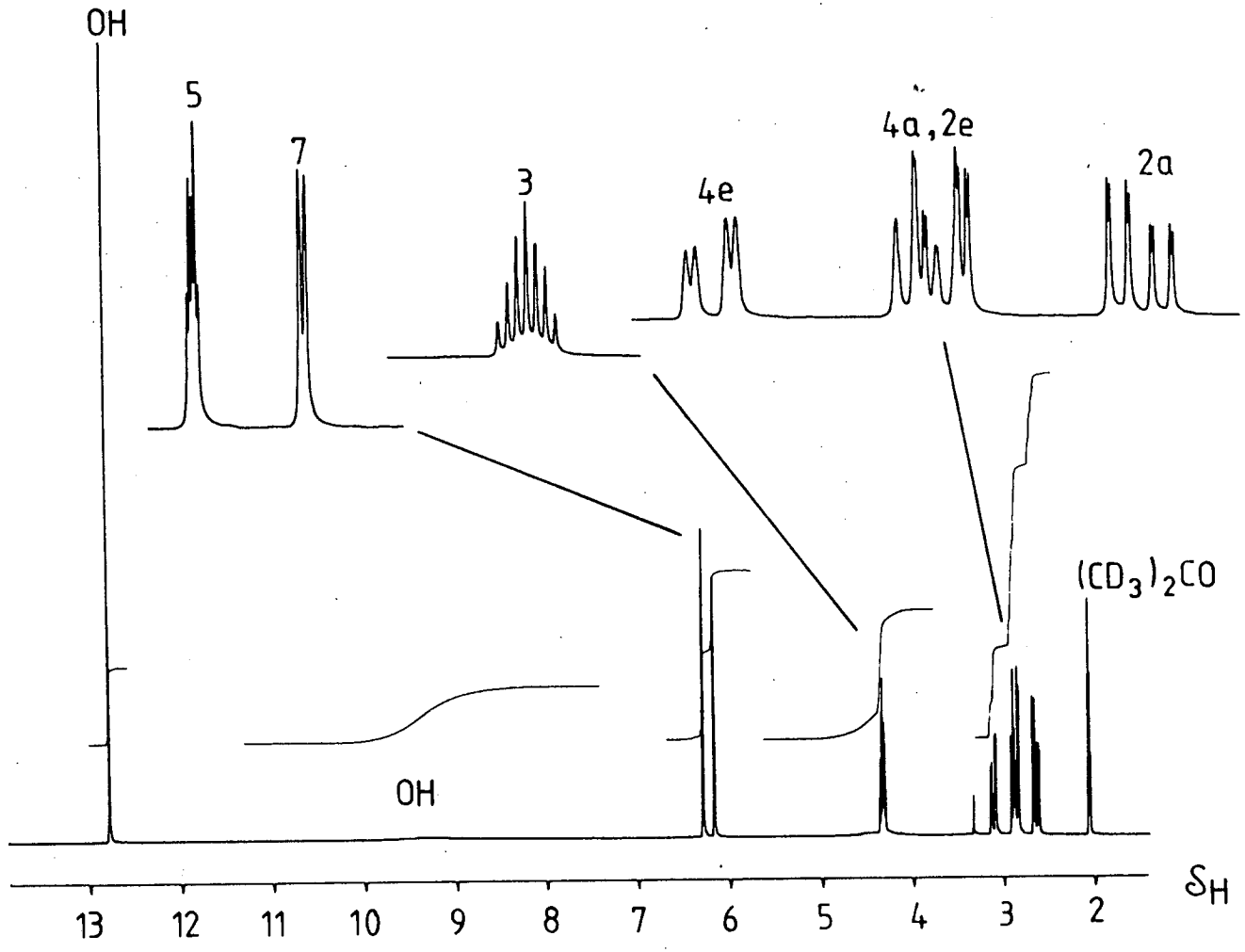


Figure 1 360 MHz ^1H n.m.r. spectrum of scytalone (1) in d_6-acetone.

TABLE 2 360 MHz ^{1}H n.m.r. spectrum of scytalone (1)

Hydrogen	H(p.p.m.)	Multiplicity	J(Hz)
7	6.15	dd	2.2, 0.6
5	6.28	dt	2.2, 1.1
3	4.31	septet	3.9
4-equat	3.20	dddd	16.1, 3.8, 1.1, 1.1
4-axial	2.86	ddddd	16.1, 7.8, 1.1, 1.0, 0.6
2-equat	2.84	ddd	17.1, 3.9, 1.0
2-axial	2.62	ddd	17.1, 7.8, 1.1

conclusions have been reported by Seto et al.[6] In a further study, incorporation of [2-^{13}C,^{2}H$_3$]acetate and examination of both proton noise-decoupled and ^{2}H noise-decoupled spectra confirmed that ^{2}H was incorporated at C-4 and C-5 only,[8] but again no ^{2}H incorporation could be observed at C-2 or C-7.

In all of the above experiments the presence of deuterium at each site of the molecule has been detected indirectly, e.g. by decreased intensity of the enriched ^{13}C signals, or by observing the ^{13}C - ^{2}H signal in ^{13}C n.m.r. spectra in which signals due to ^{13}C bearing ^{2}H show an isotope shift from the ^{13}C - ^{1}H signal. In order to obtain more information on the stereospecificity of labelling at C-4 and to study further the question of labelling at C-2 and C-7 we have used the more sensitive approach of direct ^{2}H n.m.r. We have also studied the incorporation of ^{13}C - labelled malonate to try to obtain more information on the nature of the assembly pattern of the precursor polyketide chain.

4.2 RESULTS AND DISCUSSION

A necessary prerequisite of any stable isotope labelling study is an unambiguous assignment of the n.m.r. spectrum. The ^{1}H n.m.r. spectrum of scytalone had not been rigorously assigned and indeed at normal field strengths the signals due to the C-2 and C-4 methylenes appear as complex overlapping, non-first order multiplets. However, at 360.13 MHz a full analysis of the spectrum, shown in figure 1, and assignment of all the signals was possible and revealed a wealth of long range coupling data. The observed ^{1}H chemical shifts and couplings are summarised in Table 2. These have all been confirmed by the appropriate decoupling experiments. H-7 appears as a doublet of doublets (J, 2.2 and 0.6 Hz) due to coupling to H-5 (meta coupling) and 6-bond coupling to the 4-axial hydrogen;[9] H-5 is an overlapping

TABLE 3 Production of scytalone in *P.largerbergii*

Day[a]	Crude extract(mg)[b]	Scytalone(mg)[b]
2	93	11
3	176	60
4	188	80
5	299	174
6	356	156
7	240	145
8	331	157

a Innoculated on Day 0

b Yield per flask

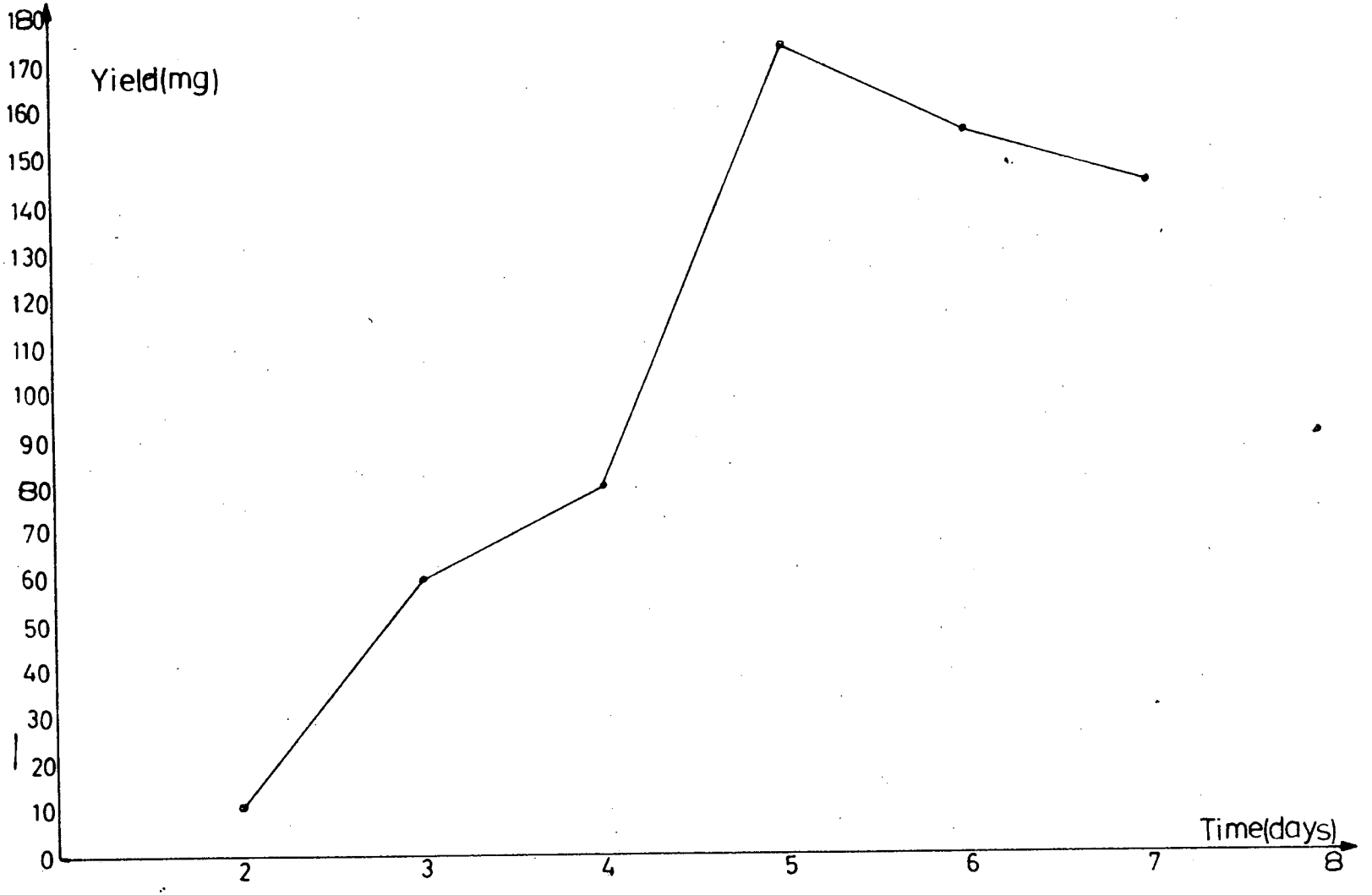


Figure 2 Production of scytalone (1) in shake cultures of P.lagerbergii.

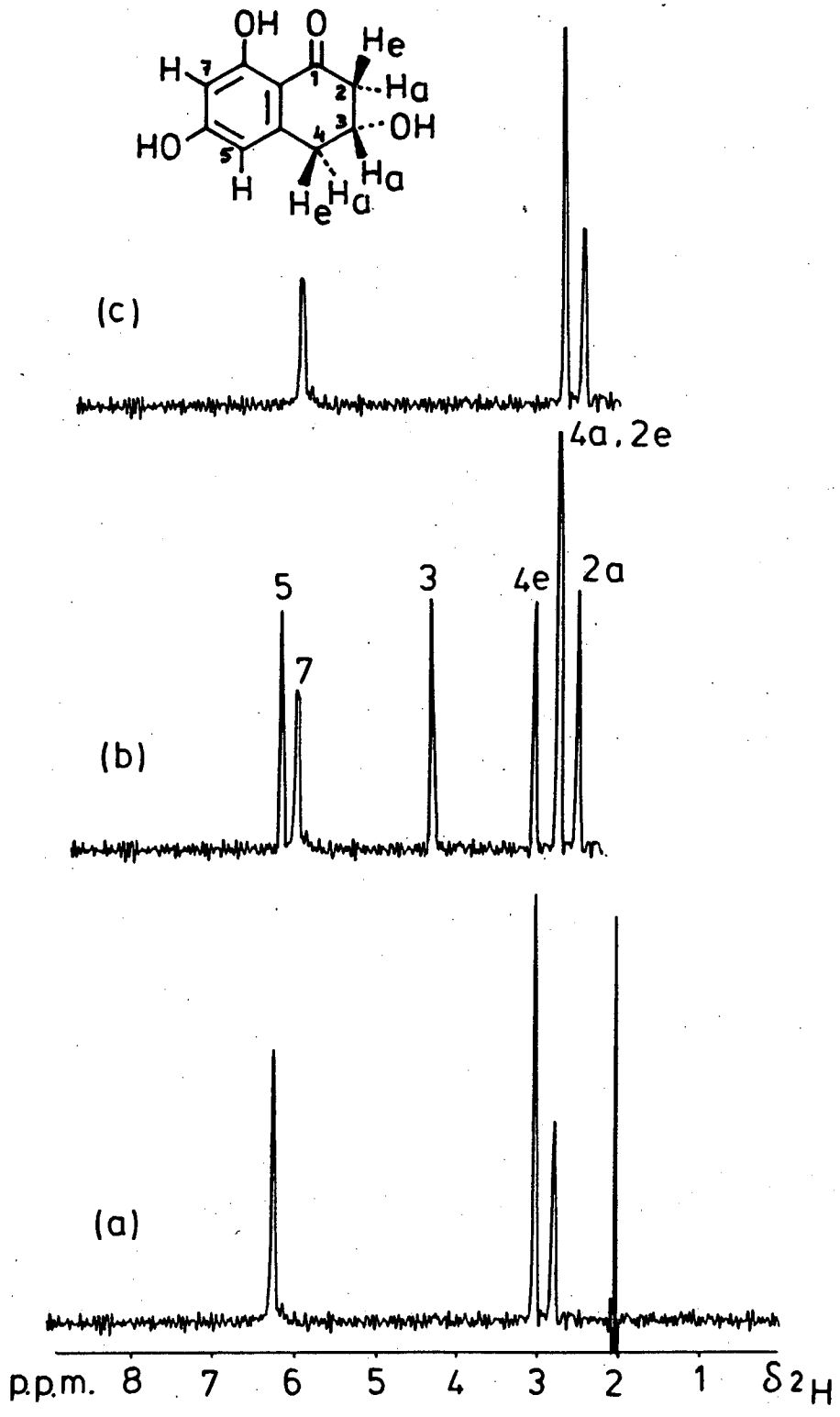


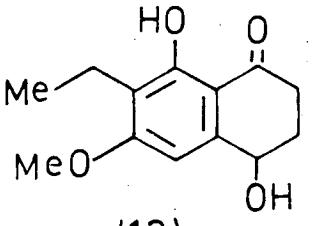
Figure 3 55 MHz ^{2}H n.m.r. spectra of scytalone (a) [$^{2}H_3$]acetate enriched, (b) uniformly H-labelled and (c) after reaction for 72 h. at room temperature in NaOMe, $MeO^{2}H$.

doublet of triplets (J, 2.2 and 1.1 Hz), the triplet splitting being due to 4-bond coupling to the benzylic methylene hydrogens. The 3-hydroxy substituent clearly occupies an equatorial orientation as H-3 appears as a symmetrical septet due to equal trans-diaxial coupling (7.8 Hz) and equal axial-equatorial couplings (3.8 Hz) to the 2-and 4-methylene hydrogens, which in turn show geminal couplings of 17.1 and 16.1 Hz respectively. In addition the equatorial hydrogens on C-2 and C-4 show a mutual 4-bond "W" coupling of 1.0 Hz and the axial hydrogens also show a mutual coupling of 1.1 Hz.

Preliminary growth experiments were carried out and these showed that production of scytalone in P. lagerbergii started on the second day of growth and finally reached a maximum on the fifth day after inoculation, these results are summarised in Table 3 and Figure 2. Preliminary feeding experiments using [1-^{14}C]acetate showed that the best day for feeding labelled precursors to P. lagerbergii was day 2. Further experiments using diethyl [2-^{14}C]malonate in ethanolic solution revealed that maximum incorporation of labelled material occurred when malonate was fed to P. lagerbergii on the third day of growth.

4.2.1 ^{2}H - Labelling Studies

P. lagerbergii was grown on a medium supplemented with [$^{2}H_{3}$]acetate and the 55.28 MHz ^{2}H n.m.r. spectrum of the isolated scytalone was determined. This showed (figure 3a) signals at δ H 2.85, 3.10 and 6.32 p.p.m. with relative intensities of 0.7, 1.6 and 1.0 respectively. The latter signals are readily assigned to the C-4 equatorial and C-5 hydrogens. However the remaining signal at 2.85 p.p.m. could be assigned to either of the 4-axial or 2-equatorial hydrogens which are not resolved in the ^{2}H n.m.r. spectrum. This was demonstrated by determining the spectrum of universally labelled



(13)

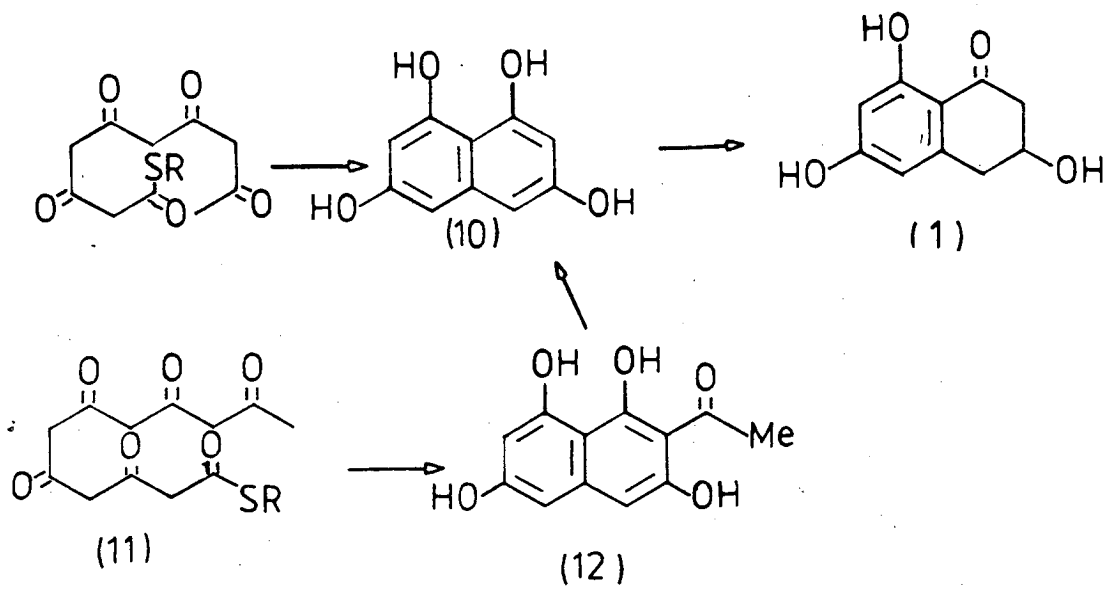
scytalone, prepared by the simple expedient of growing P. lagerbergii on a medium supplemented with 5% ^2H_2O. The spectrum, (Figure 3b) showed an equal degree of labelling at all the possible positions in scytalone.

However, this assignment problem was resolved by carrying out a series of exchange experiments in deuteriated methanol (CH_3OD) using sodium methoxide as base. It was found, (see Figure 3c) that the 2-equatorial hydrogen exchanged most rapidly, at approximately twice the rate of the 2-axial hydrogen. In addition H-7 also exchanged but about three times more slowly than the 2-equatorial hydrogen. On prolonged treatment complete exchange of H-5 was observed, but no exchange of the C-4 methylene hydrogens occurred even under forcing conditions.

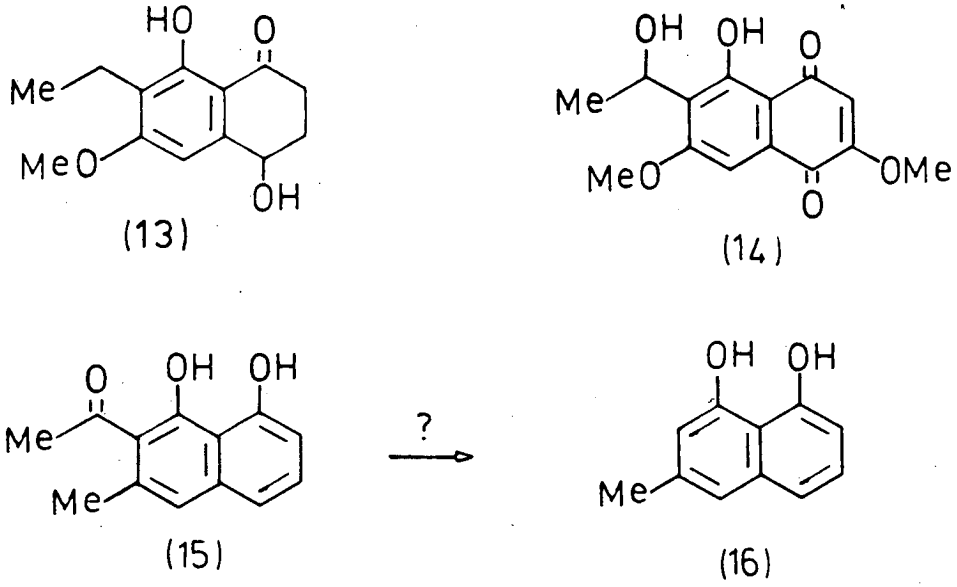
On subjecting the sample of [2-$^2H_3$]acetate enriched scytalone to a reverse exchange process using sodium methoxide and [^1H]methanol and redetermining the ^2H n.m.r. spectrum, it was apparent that no loss of ^2H label had occurred and so the signal at 2.85 p.p.m. in the spectrum of [2-$^2H_3$]acetate-enriched scytalone must be due to incorporation of ^2H at the 4-axial and not the 2-equatorial position. As previous work[5,8] has shown that only one ^2H can be incorporated at C-4, the presence of label at both the equatorial and axial positions indicates that reduction to the dihydronaphthalene occurs in a non-stereospecific manner. This contrasts with recent results obtained for O-methylasparvenone(13) where the corresponding reduction to the dihydronaphthalene is clearly stereospecific.[10] There is no incorporation of ^2H even at a low level at C-2 or C-7 of scytalone.

4.2.2 ^{13}C - Labelling Studies

In order to obtain information on the disposition of the

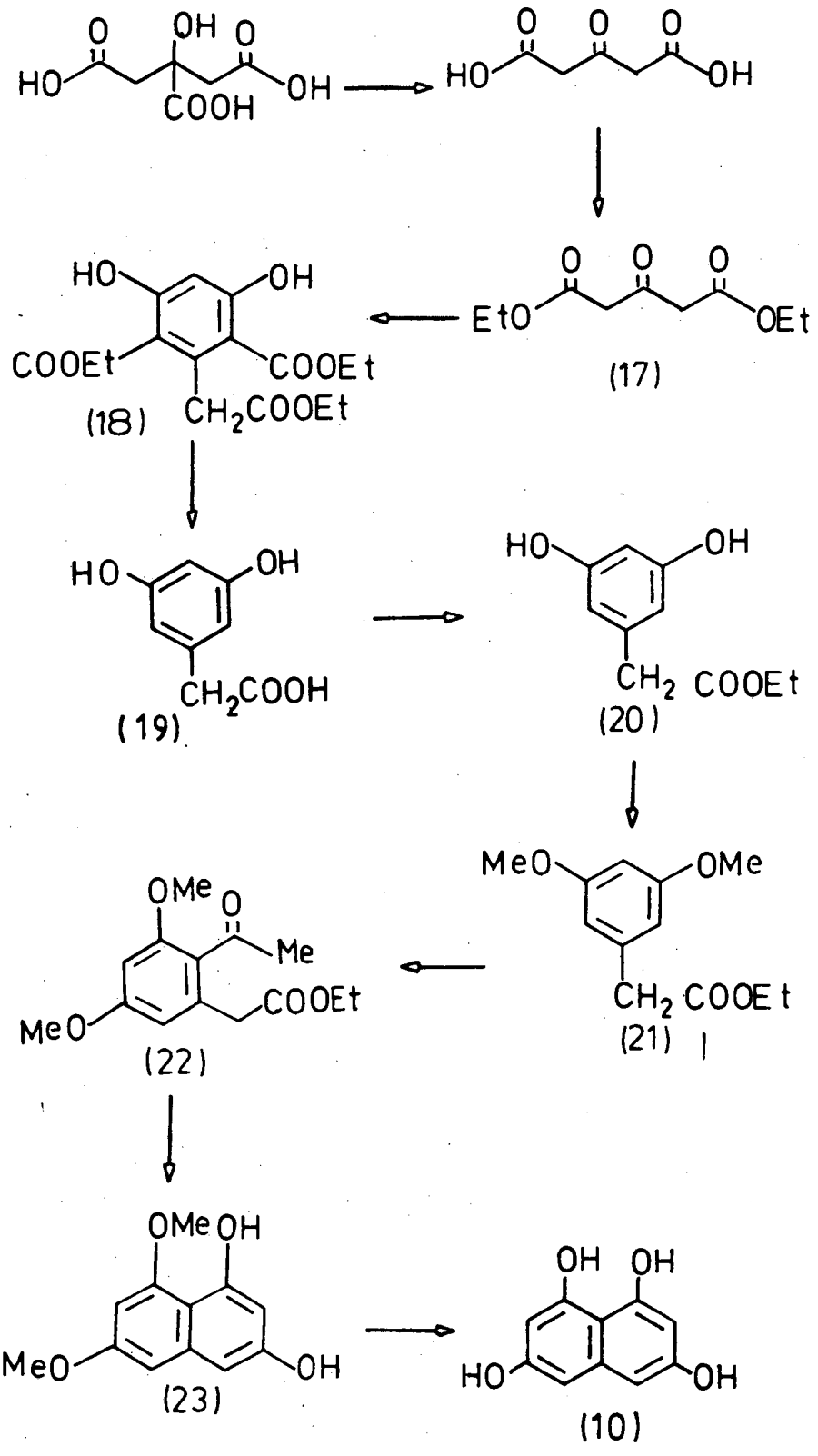

HO
OH
O
SR
HO
OH
(10)
HO
O
HO
OH
(1)
O
O
O
O
O
SR
(11)
OH
OH
O
Me
HO
OH
(12)

Scheme 4


HO
O
Me
MeO
OH
(13)
OH
OH
O
Me
MeO
OMe
O
(14)
O
OH
OH
Me
Me
(15)
?
OH
OH
Me
(16)

"Starter" acetate group of the polyketide precursor, the incorporation of [2-^{13}C]malonate into scytalone was also examined. This resulted in high enrichment (ca 5 atom %) at each of the five carbons enriched from [2-^{13}C]acetate[2] viz C-2, C-4, C-5, C-7 and C-4a but there was no discernible difference in the enrichment levels. On repeating the experiment with simultaneous addition of unlabelled sodium acetate a technique commonly used to facilitate observation of malonate starter effects,[11] the overall enrichments were lower (ca 3 atom %) but again no significant differences in enrichment levels were apparent. Although studies have been reported in which ^{2}H from acetate has been incorporated only into the acetyl-CoA derived "Starter" unit of a polyketide chain and not the malonyl-CoA derived chain extending units,[12] in a reasonably large number of studies[13] carried out in these laboratories, incorporation into both positions has been observed. These results could imply therefore, that C-4 and C-5 were partially derived from the acetate starter unit of a pentaketide precursor and so make the assembly pattern (5) shown in Scheme 2, likely, and so rule out assembly patterns (6) and (7) in Scheme 2. However, the absence of ^{2}H on C-2 and C-7 along with the failure to observe a starter effect on incorporation of [2-^{13}C]malonate can also be partially explained by proposing, as indicated in Scheme 4, that the 1,3,6,8-tetrahydroxynaphthalene(10) is formed by loss of an acetyl moiety from the corresponding 2-acetyl derivative (12) which itself would be formed from a hexaketide precursor, e.g. (11). Such hexaketide-derived naphthalene metabolites, e.g. (13) and (14) have been isolated from a number of fungi, including Scytalidium species.[14] There is also evidence that the naphthol (16) is derived by loss of the acetyl moiety from neopodin (15) in Rumex alpinus.[15]

Since incorporations of potential advanced intermediates


HO
O
OH
O
OH
COOH
HO
O
O
O
OH
EtO
O
O
O
OEt
(17)
HO
OH
COOEt
COOEt
CH2COOEt
(18)
HO
OH
CH2COOH
(19)
HO
OH
CH2 COOEt
(20)
MeO
OMe
CH2 COOEt
(21)
OMe
O
Me
MeO
COOEt
(22)
OMe OH
MeO
OH
(23)
OH
OH
HO
OH
(10)

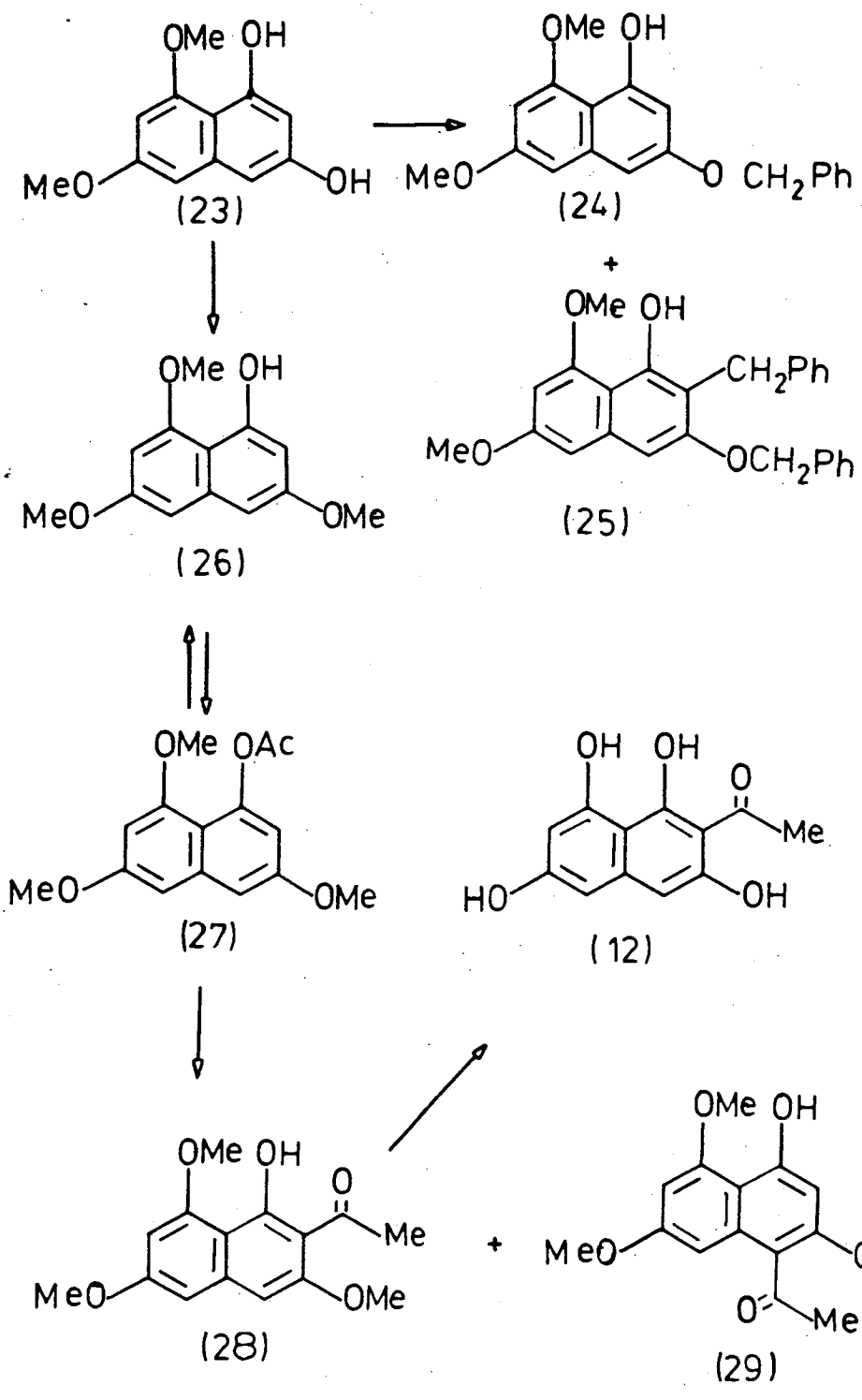
sometimes have proved invaluable in biosynthetic studies we set out to synthesise in a labelled form both 1,3,6,8-tetrahydroxynaphthalene(10) and 2-acetyl-1,3,6,8-tetrahydroxynaphthalene(12) and to test these as precursors to scytalone.

4.2.3 Synthesis of Labelled Naphthalenes

1,3,6,8-Tetrahydroxynaphthalene was prepared by modification of a previously described synthesis.[16] Acetonedicarboxylic acid was prepared[17] and converted[18] to its diethylester (17). This was condensed[19] in the presence of sodium metal at 140°C to give a 52% yield of (18) which on hydrolysis and decarboxylation gave 3,5-dihydroxyphenylacetic acid (19) which was then converted to its ethyl ester (20), and methylated with methyl iodide to give ethyl 3,5-dimethoxyphenylacetate (21) in quantitative yield.

Friedel-Crafts acylation of this compound was carried out by stirring it with acetic anhydride in the presence of boron trifluoride etherate at 60°C for two hours and gave compound (22) in 75% yield as previously described.[20] However, further investigation showed that if the above reaction mixture was stirred for a further 18 hours at room temperature, a higher yield (ca 95%) of (22) could be obtained. Treatment of (22) with sodium methoxide in refluxing methanol under an atmosphere of nitrogen afforded 1,3-dihydroxy-6,8-dimethoxy-naphthalene(23). Demethylation was achieved using hydrobromic acid in acetic acid to give the desired 1,3,6,8-tetrahydroxynaphthalene (10).

It appeared that introduction of the desired 2-acetyl substituent could be achieved by photo-Fries rearrangement[21] of the corresponding 1-acetoxynaphthalenes. To selectively acetylate the 1-hydroxyl of 1,3-dihydroxy-6,8-dimethoxynaphthalene (23), protection of the 3-hydroxyl was required. Thus (23) was reacted (stirring at room temperature for five days) with benzylbromide in acetone in the


OMe OH
MeO
OH
(23)
OMe OH
MeO
O CH2Ph
(24)
+
OMe OH
CH2Ph
MeO
OCH2Ph
(25)
OMe OH
MeO
OMe
(26)
OMe OAc
MeO
OMe
(27)
OH OH
O
Me
HO
OH
(12)
OMe OH
O
Me
MeO
OMe
(28)
+
OMe OH
MeO
OMe
O
Me
(29)

presence of potassium carbonate to investigate the use of the benzyl group for the selective protection of the 3-hydroxy group without affecting the hydrogen bonded 1-hydroxyl. Although the 1-hydroxy group remained untouched the major product of this reaction was not the desired 1-hydroxy 3-benzyloxy-6,8-dimethoxynaphthalene (24) which was formed to the extent of only 20%, but the C-benzyl compound (25), which is the result of the attack by benzyl cation on the ring, i.e. C-substitution as well as O-substitution had occurred.

Structure (25) was assigned on the basis of the ^{1}H n.m.r. spectrum which showed inter alia signals at 6.83 p.p.m. and 4.41 p.p.m. assignable to an uncoupled aromatic proton at C-4 and to benzylic methylene hydrogens respectively.

When the same reaction was repeated in refluxing acetone, a higher yield of this mixture (80%) was obtained but the yield of (24) was even poorer. However, when (23) was reacted with diazomethane, only one product (26) was formed in 60% yield. Although excess diazomethane was used, some of the starting material (ca 20%) was still present, which remained unreacted even after further addition of the reagent. When a mixture of compound (26), pyridine and acetic anhydride was stirred at room temperature for 10 hours, the acetate (27) was obtained in quantitative yield. The photo-Fries[21] rearrangement of the acetate (27) to the corresponding 1-hydroxy-2-acetyl-3,6,8-trimethoxynaphthalene (28) was attempted by irradiating compound (27) in methanol. This gave the above compound (28) as the predominant product in 60% yield along with 10% of the 4-acetyl product (29) and ca 10% of the parent phenol (26) as a by-product. Attempted demethylation by boron tribromide[22] failed to deprotect compound (28) but, hydrobromic acid in acetic acid proved to be an excellent reagent for this purpose. Thus compound (28) was

TABLE 4 Incorporation of ^{14}C-labelled naphthalenes into scytalone

Precursor	Amount fed (mg)	Sp.act. (dpm $mmol^{-1}$)	Scytalone isolated(mg)	Sp.act (dpm $mmol^{-1}$	% Incorporation
10	22.5	1.06×10^7	78	2.01×10^4	0.79
30	62.5	5.87×10^7	105	2.01×10^4	0.57

succssfully demethylated to 1,3,6,8-tetrahydroxy-2-acetylnapththalene (12) quantitatively. Careful sublimation of (12) gave an off white sold in 85% yield.

4.2.4 Incorporation of ^{14}C - Labelled Naphthalenes

On repeating the above synthetic sequence with [1-^{14}C]acetic anhydride, [1,8-^{14}C]-1,3,6,8-tetrahydroxynaphthalene (10) and [1-^{14}C]-2-acetyl-1,3,6,8-tetrahydroxynaphthalene(12) were obtained. These were fed in acetone solution to shake cultures of P. lagerbergii. Both showed significant incorporation into scytalone (Table 4), thus providing evidence for the biosynthesis of scytalone from a hexaketide precursor as shown in Scheme 4. However, as yet no degradation has been carried out to establish whether or not the precursors are incorporated intact. Synthesis of the naphthalenes (10) and (12) with a ^{13}C label at C-1 or preferably double ^{13}C labels at C-1 and C-2 could be achieved by the synthetic route above and their incorporation into scytalone studied by ^{13}C n.m.r.

4.3 EXPERIMENTAL

For general experimental conditions, see 2.8.1

Incorporation of Labelled precursors

Phialaphora lagerbergii (CMI 96745) was grown at 25°C in shaken culture in 500 ml conical flasks each containing 200 ml Czapek-Dox medium containing 0.1% yeast extract and 5% sucrose. Preliminary experiments indicated that scytalone production commenced after 4 days' growth and reached a maximum after 12-15 days' growth. Further experiments were carried out with [^{14}C]acetate and [^{14}C]malonate to determine the optimum regime for feeding ^{2}H- and ^{13}C-labelled precursors.

(a) **Incorporation of [$^{2}H_3$]acetate** Sodium [$^{2}H_3$]acetate (2g) was dissolved in distilled water (4 ml) and the sterilised

solution was distributed among eight shake flask cultures of P. lagerbergii after 5 days' growth. After a further 4 days' growth the mycelium was separated from the culture liquors by filtration and the acidified filtrate was extracted with ethyl acetate (4 x 100 ml). Evaporation of the extract gave a brown solid (1.011 g) which was purified by preparative thin layer chromatography on 20 x 20 x 0.05 cm silica GF_{254} plates eluted with 50% acetone in light petroleum (b.p. 60-80°) to give pure scytalone (600 mg).

(b) **Incorporation of [2-^{13}C]malonate** Diethyl [2-^{13}C]malonate (0.25 g) dissolved in ethanol (3 ml) was added in equal portions to one shake flask culture of P. lagerbergii after one, two and three days' growth. After a further 3 days' growth, the culture was worked up as above to give scytalone (109 mg).

(c) **Incorporation of [^{13}C]malonate in the presence of unlabelled sodium acetate** Diethyl [2-^{13}C]malonate (0.2 g) dissolved in ethanol (2 ml) was added in two equal portions to one shake flask culture of P. lagerbergii after one and two days' growth along with sodium acetate (0.25 g) in water (2.5 ml) each day. Work up as above yielded scytalone (90 mg).

(d) **Fermentation in D_2O supplemented medium** Two shake flask cultures of P.lagerbergii were grown for one day, 10 ml of culture medium was then removed from each flask and replaced by 10 ml ^2H_2O. After a further 7 days growth the cultures were worked up to give [U-^{2}H]scytalone (150 mg).

(e) **Incorporation of [1-^{14}C]-1,3,6,8-tetrahydroxy-naphthalene** [1-^{14}C]-1,3,6,8-tetrahydroxynaphthalene (10, 62.5 mg, 5.87 x 10^6 d min^{-1} $mmole^{-1}$) was dissolved in acetone (2 ml) and

added to one shake flask culture of P. lagerbergii after one and two days' growth. Work up as above yielded scytalone (105 mg, 2.01×10^4 d min^{-1} $mmole^{-1}$).

(f) **Incorporation of [1-^{14}C]-2-acetyl-1,3,6,8-tetrahydroxy-naphthalene** [1-^{14}C]-2-Acetyl-1,3,6,8-tetrahydroxynaphthalene (31, 22.5 mg, 1.06×10^7 d min^{-1}, $mmole^{-1}$) was dissolved in acetone (2 ml) and added to one shake flask of P. lagerbergii after one and two days' growth. Work up as above yielded scytalone (7.8 mg, 2.01×10^4 d min^{-1} $mmole^{-1}$).

^{2}H - Exchange Experiments

Scytalone (100 mg) was dissolved in d_4-methanol (1.5 ml) and sodium methoxide (10 mg) was added. The reaction mixture was left at room temperature and the rate of exchange monitored by ^{1}H n.m.r. After 3 days complete exchange of the 2-equatorial hydrogen and partial exchange of the 2-axial and 7-hydrogens had occurred. After 5 days, exchange of the 2-axial hydrogen was complete and leaving for 5 weeks resulted in almost complete exchange of the 5- and 7-hydrogens. After acidification and preparative t.l.c., pure scytalone (62 mg) was isolated.

Exchange of [$^2H_3$]acetate-enriched scytalone in [^{1}H]-methanol

The labelled scytalone (254 mg) was dissolved in methanol (5 ml) and sodium methoxide (30 mg) was added and the reaction mixture was left to stand for 5 days at room temperature. The mixture was then poured into water (30 ml) and the solution acidified to pH2 with dilute hydrochloric acid. Extraction with ethyl acetate (4 x 100 ml) followed by evaporation of the solvent gave the crude product (200 mg) which was purified by preparative t.l.c. to give pure scytalone (154 mg).

3,5-Dihydroxy acetic acid (19)

Fine cut pieces of sodium (0.1 g) were dissolved in diethyl-

acetonedicarboxylate (10 ml) and the solution was heated in an oil bath at 140° C for 2 hours. The thick orange coloured liquid was poured while still hot into a porcelain dish and was cooled to furnish a crude solid product which was washed several times with 50% aqueous ethanol to yield a white solid product which was recrystallised from ethanol to give colourless crystals (4.1g, 52%) of ethyl 2,6-diethoxycarbonyl-3,5-dihydroxyphenylacetate (18) m.p. 97-98°C (lit.[19] m.p. 98°C). The above product (2.5g) was gently refluxed with 28% sodium hydroxide solution (20 ml) for 2 hours. After cooling and acidification ($6MH_2SO$) the acidic solution was then concentrated on a rotary evaporator until the sodium sulphate crystallised out. After cooling the product (together with the precipitated sodium sulphate) was dissolved in ethyl acetate (150 ml), dried over magnesium sulphate and filtered. Evaporation of the solvent gave a quantitative yield of 3,5-dihydroxyphenylacetic acid (19) as a yellow oil that solidified on standing.

Ethyl 3,5-dihydroxyphenylacetate (20)

The acid (19, 2.85 g) was stirred with hydrogen chloride saturated ethanol (30 ml) for 24 hours. The solvent was evaporated and water (25 ml) was added. The aqueous solution was then extracted with ethyl acetate (3 x 10 ml). The organic solution was dried over magnesium sulphate, filtered, and evaporated to give ethyl 3,5-dihydroxyphenylacetate as an oil which was used for the next reaction without further purification. δ H 1.29 (3H, t, J 9Hz, $CH_2-C\underline{H}_3$); 3.48 (2H, s, $-C\underline{H}_2CO_2Et$); 4.15 (2H, q, J 9Hz, $C\underline{H}_2CH_3$); 6.18 (2H, d, J 2.5Hz, Ar-H); 6.30 (2H, s, exchangeable, O<u>H</u>); 6.33 (2H, d, J 2.5Hz, Ar-<u>H</u>).

Ethyl 3,5-dimethoxyphenylacetate (21)

To a solution of ethyl 3,5-dihydroxyphenylacetate (20, 196 mg)

in acetone (10 ml) was added anhydrous potassium carbonate (300 mg) and methyl iodide (350 mg). The reaction mixture was refluxed overnight, cooled to room temperature and filtered. Evaporation of the solvent gave an oil which was extracted into ethyl acetate, washed with water and dried over magnesium sulphate. Filtration and evaporation of the solvent gave a quantitative yield of practically pure ethyl 3,5-dimethoxyphenylacetate (21) b.p. 170-175°C (0.5 mm/Hg) δ H 1.23 (3H, t, J 9Hz, $CH_2\underline{CH}_3$); 3.51 (2H, s, Ar-$\underline{CH}_2$); 3.75 (6 H, s, $OC\underline{H}_3$); 4.13 (2H, q, J 9Hz, $C\underline{H}_2CH_3$); 6.36 (2H, d, J 2Hz, Ar-$\underline{H}$); 6.43 (2H, d, J 2Hz, Ar-$\underline{H}$).

Ethyl 2-acetyl-3,5-dimethoxyphenylacetate (22)

Boron tribromide (7.14 ml) was added to a mixture of ethyl 3,5-dimethoxyphenylacetate (21, 1g) and acetic anhydride (0.86 ml). The mixture was heated to 60°C for 2 hours while stirring. Heating was then stopped and the reactants were stirred for another 12 hours. The reaction mixture was then poured into water (100 ml). The aqueous solution was then extracted with ethyl acetate (3 x 50 ml). The organic solution was washed with water (3 x 20 ml) and dried over magnesium sulphate. Filtration followed by evaporation of the solvent gave ethyl 2-acetyl-3,5-dimethoxyphenylacetate (22) as a yellow oil (1.16 g, 97%) which was used for the next step without further purification. δ H 1.21 (3H, t, J 9Hz, CH_2CH_3); 2.46 (3H, s, $C\underline{H}_3CO$); 3.64 (2H, s, Ar-$\underline{CH}_2$); 3.76 (3H, s, OCH_3); 3.78 (3H, s, OCH_3); 4.18 (2H, q, J 9Hz, $C\underline{H}_2$, CH_3); 6.32 (2H, d, J 3.2Hz, Ar-H); 6.37 (2H, d, J 2.2Hz, Ar-$\underline{H}$).

1,3-Dihydroxy-6,8-dimethoxynaphthalene (23)

Ethyl 3,5-dimethoxy-2-acetylphenylacetate (22, 1.16 g) was dissolved in dry methanol (5 ml). This solution was slowly added to a refluxing solution of sodium (200 mg) in dry methanol (5 ml) under a

nitrogen atmosphere. The mixture was refluxed for 2 hours, cooled and acidified (2N H_2SO_4). The acidic solution was poured into water (100 ml) and the aqueous mixture was repeatedly extracted with chloroform. the combined extracts were washed with water and dried over magnesium sulphate. Evaporation of the solvent left the unstable 1,3-dihydroxy 6,8-dimethoxy-naphthalene (23, 0.95 g) which was used without further purification.

3-Benzyloxy-1-hydroxy-6,8-trimethoxynaphthalene (24)

To a solution of 1,3-dihydroxy-6,8-dimethoxynaphthalene (23) (950 mg) in dry acetone (10 ml) was added benzyl bromide (1.2 eq.) together with potassium carbonate (1.2 eq.). The mixture was stirred for 5 days at room temperature, filtered and the solvent was evaporated. The gummy residue was dissolved in chloroform (10 ml) and washed with water (2 x 5 ml). The organic layer was separated and dried over magnesium sulphate after which it was filtered and the solvent evaporated to give a heavily coloured residue (1.0 g). Purification of this product by t.l.c. using 40% ether in petroleum ether (40-60°) as eluent gave two bands. The band with Rf value of (0.62) gave 3-benzyloxy-1-hydroxy-6,8-dimethoxynaphthalene (24) which was recrystallised from ether-petroleum ether (30-40°) to give colourless crystals (200 mg, 20%) m.p.138-140°C (found: C, 73.42; H,5.82. $C_{19}H_{18}O_4$ requires $C_{19}H_{18}O_4$, 73.55; H, 5.81%); δH 3.96(3H, s,OC$\underline{H}_3$), 3.97 (3H, s ,OC$\underline{H}_3$); 5.10 (2H, s, ArC$\underline{H}_2$); 6.32, 6.50, 6.59, 6.62 (all 1H, d, J 2 Hz, Ar-$\underline{H}$). 7.40 (5H, m, Ar-$\underline{H}$); 9.14 (1H, s, exchangeable, O$\underline{H}$). The band with Rf 0.45 was removed to give an oil (500 mg) identified as 2-benzyl-3-benzyloxy-1-hydroxy-6,8-dimethoxy-naphthalene (25) on the basis of its n.m.r. spectrum.

δH 3.86 (3H, s, OC$\underline{H}_3$); 4.41 (2H, s, Ar C$\underline{H}_2$ Ar); 5.16 (2H, s, ArC$\underline{H}_2$O); 6.35 (1H, d, J 2.2Hz, Ar-$\underline{H}$); 6.77 (1H, d, J 2.2Hz, Ar-$\underline{H}$);

6.83 (1H, s, Ar-H); 7.18 (5H, s, Ar-H); 7.33 (5H, s, Ar-H); 9.28 (1H, s, exchangeable, OH).

1-Hydroxy-3,6,8-trimethoxynaphthalene (26)

To a cooled ($0^{o}C$) solution of 1,3-dihydroxy-6,8dimethoxynaphthalene (23, 950 mg) in dry ether (5 ml) was added diazomethane (0.30, 1.6 eq.) as an ethereal solution. The reaction mixture was stirred for five hours, after which the same amount of diazomethane was added to the mixture and the solution was stirred overnight, evaporation of the solvent gave a gummy product which after initial purification by t.l.c. using 40% ether in petroleum ether (40-60) as eluent was recrystallised from ether-petroleum ether (30-40) to give 1-hydroxy-3,6,8-trimethoxynaphthalene(26) as colourless crystals (610 mg, 60%) m.p. 90-91^{o}C (found: C, 66.54; H, 5.80 C_{13} H_{14} O_4 requires C, 66.67, H, 5.98%). δ_H 3.84 (3H, s, OCH_3); 3.86 (3H, s, OCH_3) 3.99 (3H, s, OCH_3); 6.31, 6.40, 6.58, 6.61 (all 1H, d, J 2 H , Ar-H); 9.11 (1H; s, exchangeable, OH).

1-Acetoxy-3,6,8 trimethoxynaphthalene (27)

To a solution of 1-hydroxy-3,6,8-trimethoxynaphthalene(26, 610 mg) in pyridine (4 ml) was added acetic anhydride (6 ml). The reaction mixture was stirred for 12 hours at room temperature. The mixture of the solvent then evaporated under high vacuum (0.01 mm/Hg). The resultant highly viscous oil was chromatographed (t.l.c.) in 40% either in petroleum ether (30-40^{o}) to give a crystalline product (516 mg, 71%). Recrystallisation from ether - petroleum ether (30-40^{o}) afforded pure crystals of 1-acetoxy-3,6,8-trimethoxynaphthalene(27) m.p. 110-112^{o}C (found: C, 65.8; H, 6.03, C_{15} H_{16} O_5 requires C, 65.22; H, 5.80%). δ H 2.32 (3H, s, CH_3CO); 3.85 (6H, s, 2 x OCH_3); 6.35, 6.60, 6.63, 6.90 (all 1H, d, J 2.5Hz, ArH).

2-Acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (28)

The solution of 1-acetoxy-3,6,8-trimethoxynaphthalene(27, 516 mg) in methanol (150 ml) which had been purged with nitrogen for 2 hours was irradiated under N_2 for 24 hours with a 400 Watt medium pressure mercury lamp. The solvent was removed to give a pale brown oil which was chromatographed (t.l.c.) on silica gel with benzene/ether/formic acid (95,4,1). After a second elution the major yellow band separated and was eluted with chloroform to furnish 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (28) as a highly crystalline yellow product (300 mg, 58%).

m.p. 143-144°C (Found: C, 64.99; H, 5.64; $C_{12}H_{16}O_5$ requires C 65.22; H, 5.80%)

δ_H 2.65 (3H, s, CH_3CO); 3.87 (3H, s, OCH_3); 3.89 (3H, s, OCH_3); 3.92 (3H, s, OCH_3); 6.31 (1H, d, J 2.2Hz, ArH); 6.37 (1 H, s, Ar-H); 6.48 (1H, d, J 2.2Hz, ArH); 15.31 (1 H, s, exchangeable, OH).

2-Acetyl-1,3,6,8-tetrahydroxynaphthalene (12)

2-Acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (28, 300 mg) was dissolved in glacial acetic acid (16 ml). After flushing the reaction flask with nitrogen hydrobromic acid (6 ml) was added. This mixture was then refluxed under an atmostphere of nitrogen for 3 hours. The mixture was cooled to room temperature and the solvent was removed (in vacuo) after which water (20 ml) was added to the dark residue and extracted with ethyl acetate (2 x 10 ml). The organic solution was then washed with water (2 x 5 ml) dried over $MgSO_4$ (under nitrogen) filtered and the solvent was evaporated to furnish a dark brown solid (245 mg, 97%) subsequent sublimation of this crude product at 150°C/0.01 mm/Hg gave 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (12) as an off-white solid (200 mg) (found: C, 61.76; H 4.53; $C_{12}H_{10}O_5$ requires C 61.49; H 4 27%).

δ_H 2.06 (3 H, s, COMe); 6.23-6.46(3H, m, Ar-H).

1,3,6,8-Tetrahydroxynaphthalene (10)

1,3-Dihydroxy-6,8-dimethoxynaphthalene(23, 900 mg) was dissolved in acetic acid (20 ml) under an atmosphere of nitrogen and hydrobromic acid (6 ml) was added to this solution. The mixture was heated under reflux for 3 hours. Cooled to room temperature and worked up as for 1,3,6,8 tetrahydroxy-2-acetylnaphthalene, to give a gummy, heavily coloured residue which was purified (t.l.c.) using 60% acetone in n-hexane as eluent. The band Rf 0.52 was separated and washed with ethylacetate. Removal of solvent gave 1,3,6,8-tetrahydroxynaphthalene (10). δ H 6.20-6.46 (4H, m, ArH).

[1-^{14}C]-1,3,6,8-Tetrahydroxynaphthalene (10) and

[1-^{14}C]-2-acetyl-1,3,6,8-Tetrahydroxynaphthalene (12)

These were prepared exactly as described above starting with ethyl 3.5-dimethoxyphenylacetate (21) and [1-^{14}C]acetic anhydride. In this way [1-^{14}C]-1,3,6,8-tetrahydroxynaphthalene and [1-^{14}C]-2-acetyl-1,3,6,8-tetrahydroxynaphthalene were prepared with specific activities of 5.87×10^6 d min^{-1} $mmol^{-1}$ and 1.06×10^7 d min^{-1} $mmol^{-1}$.

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Biosynthesis of Tajixanthone in *Aspergillus variecolor*; Incorporation of [$^2H_3$]Acetate and [1,2-$^{13}C_2$]Acetate

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Biosynthesis of Tajixanthone in *Aspergillus variecolor*; Incorporation of [$^2H_3$]Acetate and [1,2-$^{13}C_2$]Acetate

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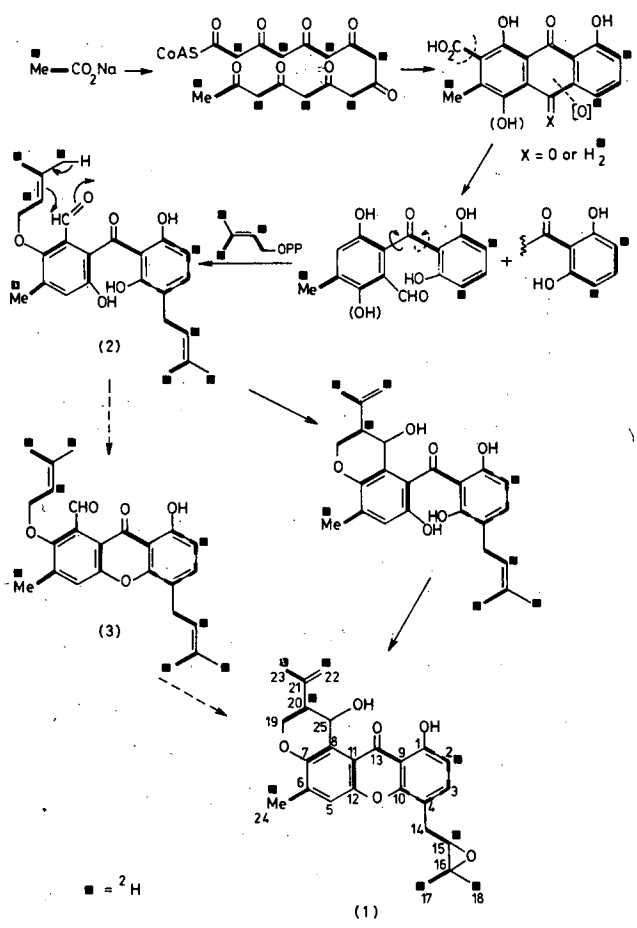
Summary The results of ^{13}C and ^2H n.m.r. analyses of [$^2H_3$]- and [1,2-$^{13}C_2$]-acetate-enriched tajixanthone are reported, which indicate, *inter alia*, that ring cleavage of an anthraquinone, and not anthrone, precursor must precede *C*-prenylation, and that dihydropyran ring formation precedes xanthone ring formation during biosynthesis of tajixanthone.

Two major biosynthetic pathways are evident in *Aspergillus variecolor*, one leading to tajixanthone (**1**) and related mycelial pigments,[1] and the other to andibenin and related compounds isolated from the culture liquors.[2] Previous studies[3] have indicated that biosynthesis of tajixanthone occurs *via* an octaketide-derived anthrone or anthraquinone with the introduction of two prenyl units from 3,3-dimethylallylpyrophosphate (DMAPP) to give an *O*-prenyloxyaldehyde intermediate (**2**), which then undergoes an intramolecular 'ene' reaction to form the substituted dihydropyran ring, and cyclodehydration to form the xanthone system. Further indirect evidence in support of this pathway was given by a detailed study of the metabolites of a number of variant strains of *A. variecolor*,[1] but the sequence and mechanistic details of the required steps remained to be determined. We now report the results of incorporation studies with [1,2-$^{13}C_2$]- and [2-$^2H_3$]-acetate which, in conjunction with ^{13}C and ^2H n.m.r. data, allow some of these details to be elucidated.

The labelling patterns resulting from these incorporation studies† are summarised in the Scheme and the following conclusions can be drawn from the ^{13}C–^{13}C labelling pattern. (i) The acetate assembly pattern in the xanthone system is entirely consistent with an octaketide precursor folded as shown in the Scheme; *cf.* islandicin.[4] (ii) The randomisation of labelling in ring c means that ring c must have been symmetrical and free to rotate on the enzyme surface at some stage in the biosynthesis of tajixanthone. This means that ring cleavage of the carbocyclic precursor must precede introduction of the *C*-prenyl residue; *cf.* ravenelin.[5] (iii)



SCHEME

† The details of the ^{13}C and ^2H n.m.r. data will be published elsewhere.

C-Prenylation and epoxidation, in agreement with recent studies on echinulin[6] and flavoglaucin,[7] occurs with retention of configuration about the double bond of DMAPP. (iv) The stereospecificity of labelling in the dihydropyran ring suggests its formation from the *O*-prenylaldehyde moiety by a *concerted* 'ene' reaction. The transition state necessary for the observed 20,25-*trans* stereochemistry of (**1**) in a concerted reaction requires dihydropyran ring formation to occur before cyclodehydration to the xanthone system, as the transition state necessary for *trans* stereochemistry in an 'ene' reaction of the xanthone aldehyde (**3**) would have a highly unfavourable interaction between the aldehyde and xanthone carbonyls. Indeed, *in vitro* cyclisation of (**3**) gives the *cis* product.[8]

^{2}H N.m.r. spectroscopy has been successfully applied to the study of terpenoid biosynthesis but so far only to a few polyketide problems.[9] The apparent intermediacy of the aldehyde (**2**) in tajixanthone biosynthesis suggests that ring cleavage might occur at the anthrone rather than anthraquinone oxidation level, in which case H-25 would be derived from the hydrogen of acetate. As the 25-hydroxy-group of (**1**) is known to be resistant to reaction, *e.g.* oxidation, a ^{2}H-labelling study seemed appropriate. The ^{2}H n.m.r. spectrum of [$^2H_3$]acetate-enriched tajixanthone indicated the labelling pattern shown in the Scheme and permits the following conclusions. (i) There is no ^{2}H label on C-25; this implies cleavage of an anthraquinone rather than an anthrone intermediate. (ii) The absence of ^{2}H on C-5 indicates that decarboxylation of the octaketide precursor occurs *after* cyclisation and aromatisation. (iii) A differential incorporation of ^{2}H is apparent. The DMAPP-derived positions are enriched to a greater extent than the polyketide methyl which appears to be more highly enriched than the polyketide methylene positions. Indeed it is reassuring to see the presence of label on C-2 as, in two recent studies, ^{2}H was incorporated only into the acetyl coenzyme A-derived position and not those derived from malonyl coenzyme A.[10,11] (iv) There appears to be some loss of ^{2}H from the *E* methyl group relative to the *Z* methyl group of DMAPP (after allowing for the anticipated 2:3 ratio). This could be occurring from acetoacetyl coenzyme A, or could be due to dimethylallyl–isopentenyl pyrophosphate equilibration.

The above results allow the sequence of steps shown in the Scheme to be proposed for the biosynthesis of tajixanthone. Further studies to delineate the pathway are in progress and will be reported in due course.

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^{13}C AND ^{2}H LABELLING STUDIES ON THE BIOSYNTHESIS OF SCYTALONE IN *PHIALAPHORA LAGERBERGII*

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(*Received in USA* 15 *October* 1982)

Abstract—The regio- and stereospecificity of incorporation of label from [$^{2}H_3$] acetate into scytalone, a dihydronaphthalene metabolite of *Phialaphora lagerbergii*, has been determined by high field ^{1}H and ^{2}H NMR studies. Incorporation studies with [2-^{13}C] malonate have failed to reveal an acetate "starter" effect suggesting that scytalone may be derived from a hexaketide precursor rather than a pentaketide as previously proposed.

Scytalone (**1**) is a dihydronaphthalene metabolite produced by a number of fungi including a *Scytalidium sp.*,[1] *Phialaphora lagerbergii*,[2] and *Verticillium dahliae*[3] in which it has been shown to be an intermediate on the pathway to fungal melanins.[4] A number of biosynthetic studies using ^{13}C and ^{2}H labelled precursors have been reported. Incorporation of singly labelled ^{13}C-acetates suggested a pentaketide origin for scytalone,[2] and studies with [1, 2-$^{13}C_2$]acetate[5,6] showed a randomisation of ^{13}C–^{13}C couplings consistent with scytalone being biosynthesised *via* a symmetrical intermediate, 1,3,6,8-tetrahydroxynaphthalene (**2**), as indicated in the scheme, and this compound can indeed be converted *in vitro* to scytalone by sodium borohydride reduction.[7] Incorporation of [2-^{13}C, $^{2}H_3$]acetate and examination of both proton noise-decoupled and ^{2}H noise-decoupled spectra indicated that ^{2}H was incorporated at C-4 and C-5 only.[8] Surprisingly no ^{2}H incorporation could be observed at C-2 or C-7. In order to obtain more information on the stereospecificity of labelling at C-4 and to study further the question of labelling at C-2 and C-7 we have used the more sensitive approach of direct ^{2}H NMR. We have also studied the incorporation of ^{13}C-malonate to try to obtain more information on the nature of the assembly pattern of the precursor polyketide chain.

A necessary prerequisite of any stable isotope labelling study is an unambiguous assignment of the NMR spectrum. The ^{1}H NMR spectrum of scytalone had not been rigorously assigned and indeed at normal field strengths the signals due to the C-2 and C-4 methylenes appear as complex overlapping, non-first order multiplets. However at 360.13 MHz a full analysis of the spectrum and assignment of all the signals was possible and revealed a wealth of long-range coupling data. The ^{1}H chemical shifts and couplings are summarised in the Table. These have all been confirmed by the appropriate decoupling experiments. H-7 appears as a doublet of doublets (J, 2.2 and 0.6 Hz) due to coupling to H-5 and a 6-bond coupling to the 4-axial hydrogen;[9] H-6 as an overlapping doublet of triplets (J, 2.2 and 1.1 Hz) the triplet splitting being due to 4-bond coupling to the 4-methylene hydrogens. The 3-OH substituent clearly occupies an equatorial orientation as H-3 appears as a symmetrical septet due to equal trans diaxial couplings (7.8 Hz) and equal axial-equatorial couplings (3.8 Hz) to the 2- and 4-methylene hydrogens, which in turn show geminal couplings of 17.1 and 16.1 Hz respectively. In addition the equatorial hydrogens on C-2 and C-4 show a mutual 4-bond "W" coupling of 1.0 Hz, and the axial hydrogens also show a mutual coupling of 1.1 Hz.

P. lagerbergii was grown on a medium supplemented with [$^{2}H_3$]acetate and the 55.28 MHz ^{2}H NMR spectrum of the isolated scytalone was determined. This showed, Fig. 1(a), signals at δ_H 2.85, 3.10 and 6.32 ppm with relative intensities of 0.7, 1.6 and 1.0 respectively. The latter signals are readily assigned to the 4-equatorial and 5-hydrogens. However the remaining signal at 2.85 ppm could be assigned to either of the 4-axial or 2-equatorial hydrogens which are not resolved in the ^{2}H NMR spectrum. This was demonstrated by determining the spectrum of universally-labelled scytalone, prepared by the simple expedient of growing *P. lagerbergii* on a medium supplemented with 5% $^{2}H_2O$. The spectrum, Fig. 1(b), showed an equal degree of labelling at all the possible positions in scytalone. However the problem was resolved by carrying out a series of exchange experiments in deuteriated methanol using sodium methoxide as base. It was found, see Fig. 1(c), that the 2-equatorial H exchanged most rapidly, at approximately twice the rate of the 2-axial H. In addition the 7-H exchanged about three times more slowly than the 2-equatorial H and on prolonged treatment complete exchange of the 5-H was also obtained. However no exchange of the 4-H's occurred even under forcing conditions. On subjecting the sample of [2-$^{2}H_3$]acetate enriched scytalone to a reverse exchange process using sodium methoxide and [^{1}H]methanol and redetermining the ^{2}H NMR spectrum it was apparent that no loss of ^{2}H label had occurred, and so the signal at 2.85 ppm in the spectrum of [2-$^{2}H_3$]acetate-enriched scytalone must be due to incorporation of ^{2}H at the 4-axial and not the 2-equatorial position. As previous work[8] has shown that only one ^{2}H can be incorporated at C-4 the presence of label at both the equatorial and axial positions indicates that reduction to the dihydronaphthalene occurs in a non-stereospecific manner. This contrasts with recent results for O-methyl aspar-

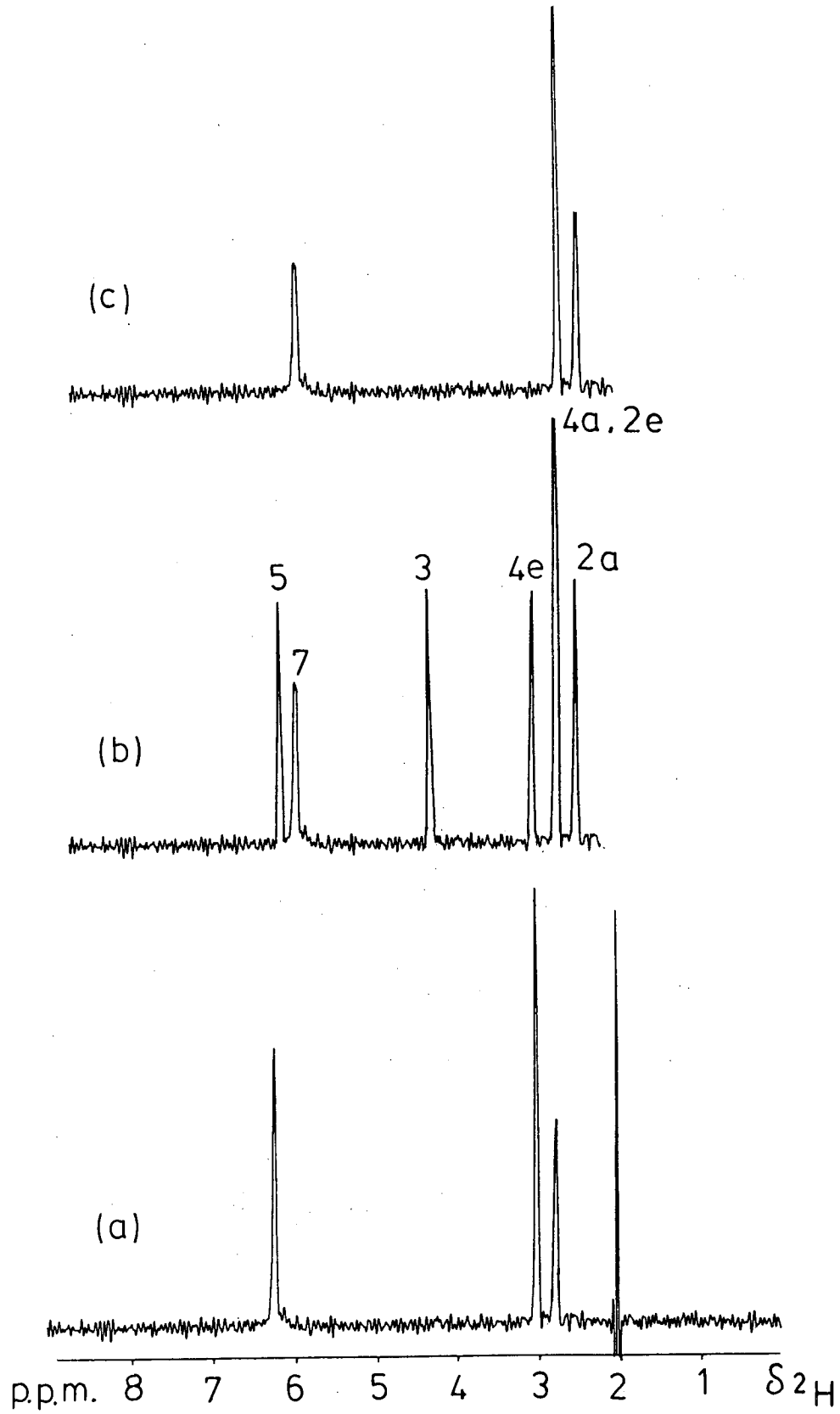
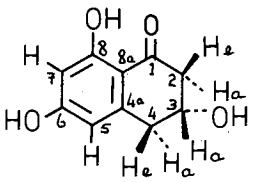


Fig. 1. 55.28 MHz NMR spectra of scytalone (a) [$^2H_3$]acetate enriched; (b) [U-^{2}H]-labelled; (c) after reaction for 72 hr at room temperature in NaOME, MeO^{2}H. All spectra were determined with proton noise-decoupling using 1000 Hz sweep widths, 2 K data points acquired and transformed into 16 K data points, pulse width 23 μs, acquisition time 1.0 s, line broadening −2.5 Hz, gaussian multiplier 0.40.

Table 1. ^{1}H NMR data for scytalone (1)

Hydrogen	δ_H(ppm)	Multiplicity	J (Hz)
7	6.15	dd	2.2, 0.6
5	6.28	dt	2.2, 1.1
3	4.31	septet	3.9
4-equat	3.20	dddd	16.1, 3.8, 1.1, 1.1
4-axial	2.86	ddddd	16.1, 7.8, 1.1, 1.0, 0.6
2-equat	2.84	ddd	17.1, 3.9, 1.0
2-axial	2.62	ddd	17.1, 7.8, 1.1



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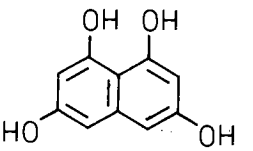

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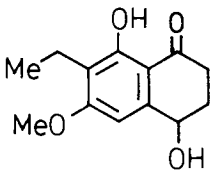

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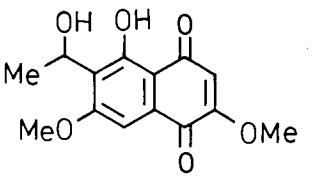

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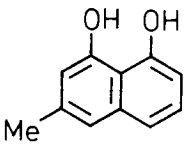

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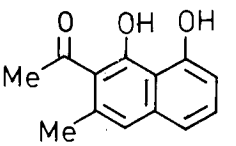

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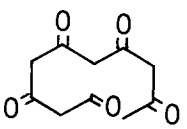

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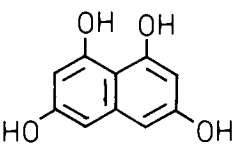

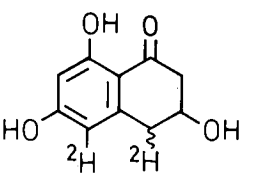

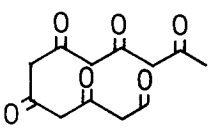

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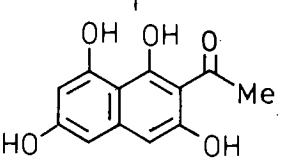

(9)

Scheme 1. Alternative pentaketide and hexaketide biosynthetic pathways to scytalone.

venone (**3**) where the corresponding reduction is clearly stereospecific.[10] Thus there is no incorporation of ^2H, even at a low level on C-2 or C-7 of scytalone.

In order to obtain information on the disposition of the "starter" acetate group of the polyketide precursor the incorporation of [2-^{13}C]malonate into scytalone was also examined. This resulted in high enrichment (*ca* 5 at.%) at each of the five carbons enriched from [2-^{13}C]acetate,[2] *viz* C-2, C-4, C-5, C-7 and C-8a but there was no discernible difference in enrichment levels. On repeating the experiment with simultaneous addition of unlabelled sodium acetate, a technique commonly used to facilitate observation of malonate "starter" effects,[11] the overall enrichments were lower (*ca* 3 at.%) but again no significant differences in enrichment levels were apparent.

These ^2H and ^{13}C labelling results are open to two possible interpretations. Although studies have been reported in which ^2H from acetate has been incorporated only into the acetyl-CoA derived "starter" unit of a polyketide chain and not into the malonyl CoA derived chain extending units,[12] we ourselves, in a reasonably large number of studies to date have always observed incorporation into both positions.[13] However, this could imply that C-4 and C-5 were partially derived from the acetate "starter" unit of a pentaketide precursor and so make assembly pattern (**7**) likely. However the absence of ^2H on C-2 and C-7 can be partially explained along with the failure to observe a "starter" effect on incorporation of [2-^{13}C]malonate by proposing that the 1,3,6,8-tetrahydroxynaphthalene (**2**) is formed by loss of an acetyl moiety from the corresponding 2-acetyl-derivative (**9**), which itself would be formed from a *hexaketide* precursor e.g. **8**. Such hexaketide-derived naphthalene metabolites e.g. **3** and **4** have been isolated from a number of fungi, including *Scytalidium* species.[14] There is also evidence that the naphthol (**5**) is derived by loss of the acetyl moiety from nepodin (**6**) in *Rumex alpinus*.[15]

Studies with potential advanced intermediates to further test these proposals are in progress.

EXPERIMENTAL

NMR spectra were determined on a Bruker WH 360 spectrometer: ^1H NMR spectra at 360·13 MHz in hexadeuterioacetone solns; ^2H NMR spectra at 55.28 MHz in acetone solns; and ^{13}C NMR spectra at 90.56 MHz in hexadeuterioacetone solns.

Incorporation of labelled precursors. Phialaphora lagerbergii (CMI 96745) was grown at 25° in shaken culture in 500 ml conical flasks each containing 200 ml Czapek-Dox medium containing 0.1% yeast extract and 5% sucrose. Preliminary experiments indicated that scytalone production commenced after 4 days' growth and reached a maximum after 12–15 days' growth. Further experiments were carried out with [^{14}C]acetate and [^{14}C]malonate to determine the optimum regime for feeding ^2H- and ^{13}C-labelled precursors.

(a) *Incorporation of* [$^2H_3$]*acetate.* Sodium [$^2H_3$]acetate (2 g) was dissolved in distilled water (4 ml) and the sterilised soln was distributed among eight shake flask cultures of *P. lagerbergii* after 5 days' growth. After a further 4 days' growth the mycelium was separated from the culture liquors by filtration and the acidified filtrate was extracted with ethyl acetate (4 × 100 ml). Evaporation of the extract gave a brown solid (1.011 g) which was purified by preparative thin layer chromatography on 20 × 20 × 0.05 cm silica GF_{254} plates eluted with 50% acetone in light petroleum (b.p. 60–80°) to give pure scytalone (600 mg).

(b) *Incorporation of* [2-^{13}C]*malonate.* Diethyl [2-^{13}C]malonate (0.25 g) dissolved in EtOH (3 ml) was added in equal portions to one shake flask culture of *P. lagerbergii* after one, two and three days' growth. After a further 3 days' growth, the culture was worked up as above to give scytalone (109 mg).

(c) *Incorporation of* [^{13}C]*malonate in the presence of unlabelled sodium acetate.* Diethyl [2-^{13}C]malonate (0.2 g) dissolved in ethanol (2 ml) was added in two equal portions to one shake flask culture of *P. lagerbergii* after one and two days' growth along with sodium acetate (0.25 g) in water (2.5 ml) each day. Work up as above yielded scytalone (90 mg).

(d) *Fermentation in* D_2O *supplemented medium.* Two shake flask cultures of *P. lagerbergii* were grown for one day, 10 ml of culture medium was then removed from each flask and replaced by 10 ml ^2H_2O. After a further 7 days' growth the cultures were worked up to give [*U*-^{2}H]scytalone (150 mg).

^2H *exchange experiments.* Scytalone (100 mg) was dissolved in d_4-methanol (1.5 ml) and NaOMe (10 mg) was added. The mixture was left at room temp and the rate of exchange monitored by ^1H NMR. After 3 days complete exchange of the 2-equatorial H and partial exchange of the 2-axial and 7-H's had occurred. After 5 days, exchange of the 2-axial H was complete and leaving for 5 weeks resulted in almost complete exchange of the 5- and 7-H's. After acidification and preparative TLC, pure scytalone (62 mg) was isolated.

Exchange of [$^2H_3$]*acetate-enriched scytalone in* [^{1}H]-*methanol.* The labelled scytalone (254 mg) was dissolved in MeOH (5 ml) and NaOMe (30 mg) was added and the mixture was left to stand for 5 days at room temp. The mixture was then poured into water (30 ml) and the soln acidified to pH 2 with dil HCl. Extraction with EtOAc (4 × 100 ml) followed by evaporation of the solvent gave the crude product (200 mg) which was purified by preparative TLC to give pure scytalone (154 mg).

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Studies on a Synthesis of (*RS*)-Mevalonic Acid Lactone

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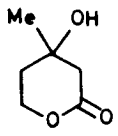
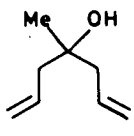
Full details of a high yielding synthesis of mevalonic acid lactone (**1**) which is of particular value in the preparation of 3- and/or 3'-labelled compounds are described. The key step, conversion of 3-hydroxy-3-methylpentane-1,5-dioic acid (**3**) into 3-hydroxy-3-methylpentane-1,5-dioic anhydride (**4**) using acetic anhydride, has been fully investigated, and an additional method using acetyl chloride and triethylamine is described.

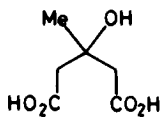
Although a number of methods for the synthesis of isotopically labelled mevalonic acid lactone (**1**) have been described in recent years,[1-5] there are a few applicable to the synthesis of the 3'- and/or 3-labelled compounds. A short, high yielding synthesis of mevalonic acid lactone which enabled isotopic labels to be introduced into these positions was recently described.[6] In this synthesis ethyl acetate was treated with allylmagnesium bromide to give 4-hydroxy-4-methylhepta-1,6-diene (**2**) which was then converted by ozonolysis and further treatment of the crude product with hydrogen peroxide in acetic acid into 3-hydroxy-3-methylpentane-1,5-dioic acid (**3**). This in turn was converted into the corresponding anhydride (**4**) by being stirred with an 'excess' of acetic anhydride at room temperature, followed by sodium borohydride reduction of the anhydride to give mevalonic acid lactone (**1**). By starting with ethyl [2-^{13}C]acetate, [3'-^{13}C]mevalonic acid lactone could be obtained in 58% overall yield. As some problems with this synthetic procedure have been reported[7] we now describe full details of the procedure along with further studies to account for the reported problems, and a modification to facilitate the use of labelled starting materials.



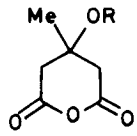
(**1**)



(**2**)



(**3**)



(**4**) R = H
(**5**) R = Ac

Isotopically labelled acetate is more readily available and more conveniently handled as the sodium salt than the ethyl ester. The sodium salt can be converted into the ethyl ester in high yield by treatment with an excess of triethyl phosphate. However, separation of the ethyl acetate from the triethyl phosphate requires careful fractional distillation and it is more convenient to use tri-n-butyl phosphate and convert the sodium acetate into the corresponding n-butyl acetate.[8]

The Grignard reaction to convert ethyl (or n-butyl) acetate into (**2**) and its further conversion into the diacid (**3**) proceeds without difficulty and requires no further comment. However, problems appear to have been encountered in the next step, in which the diacid (**3**) is converted into the corresponding anhydride (**4**) by treatment with acetic anhydride. In a recent study,[7] the diacid (**3**) was found to be converted quantitatively into 3-acetoxy-3-methylpentane-1,5-dioic anhydride (**5**) with the desired product (**4**) being formed only as a transient intermediate. However, on systematic re-examination of the reaction, we find that the product formed depends on the relative concentrations of the diacid (**3**) and acetic anhydride, and on the reaction temperature. If fewer than 20 equivalents of acetic anhydride are used and the reaction temperature is below 30 °C the desired anhydride (**4**) is formed consistently in essentially quantitative yield. In a typical experiment, a solution of the diacid (**3**) (405 mg, 2.5 mmol) in acetic anhydride (3.5 ml, 35 mmol) was stirred at 18 °C. The course of the reaction was monitored by withdrawing small aliquots of the reaction mixture, removing the acetic anhydride immediately under reduced pressure, and determining the 80 MHz ^{1}H n.m.r. spectrum. As shown in Figure 1 the acid was converted smoothly into anhydride (**4**) with no trace of the acetate (**5**). After 24 h the initial suspension had turned into a clear solution. The excess of acetic anhydride was then removed to give a white solid shown by ^{1}H n.m.r. spectroscopy [Figure 2(a)] to be essentially pure anhydride (**4**). On repeating the reaction using the same batch of acid, but on double the scale, approximately 48 h were required for complete reaction. However, the time taken for complete reaction varied with the batch of acid used [normally the diacid (**3**) obtained from the ozonolysis step is used without further purification].

If 25 or more equivalents of acetic anhydride are used, the product is indeed the acetate (**5**) [Figure 2(b)]. Increasing the temperature to 30 °C (14 equivalents of acetic anhydride) still resulted in the anhydride (**4**) as the major product, but at 40 °C the acetate (**5**) was formed along with (**4**) as a 2:1 mixture. Interestingly, heating the diacid (**3**) to 100 °C with only 1.2 equivalents of acetic anhydride again gave anhydride (**4**) as the sole product.

Although we find the use of acetic anhydride the most convenient method for the production of anhydride (**4**), an alternative method not subject to the same variability is to react the diacid with acetyl chloride and triethylamine in tetrahydrofuran (THF) at 0 °C. Reaction is again quantitative and is complete in only 2 h. The acetate (**5**) can be more conveniently prepared from the diacid (**3**) by heating in acetyl chloride at 50 °C for 4 h. Removal of excess of acetyl chloride gives a quantitative yield of (**5**). Anhydride (**4**) can also be converted readily into the corresponding acetate (**5**) by being stirred at room temperature with a slight excess of acetyl chloride.

Treatment of the anhydride (**4**) with sodium borohydride in propan-2-ol, acidification, and continuous extraction with ether gave almost pure mevalonic acid lactone which was purified finally by column chromatography on silica using hexane–ether as eluant.

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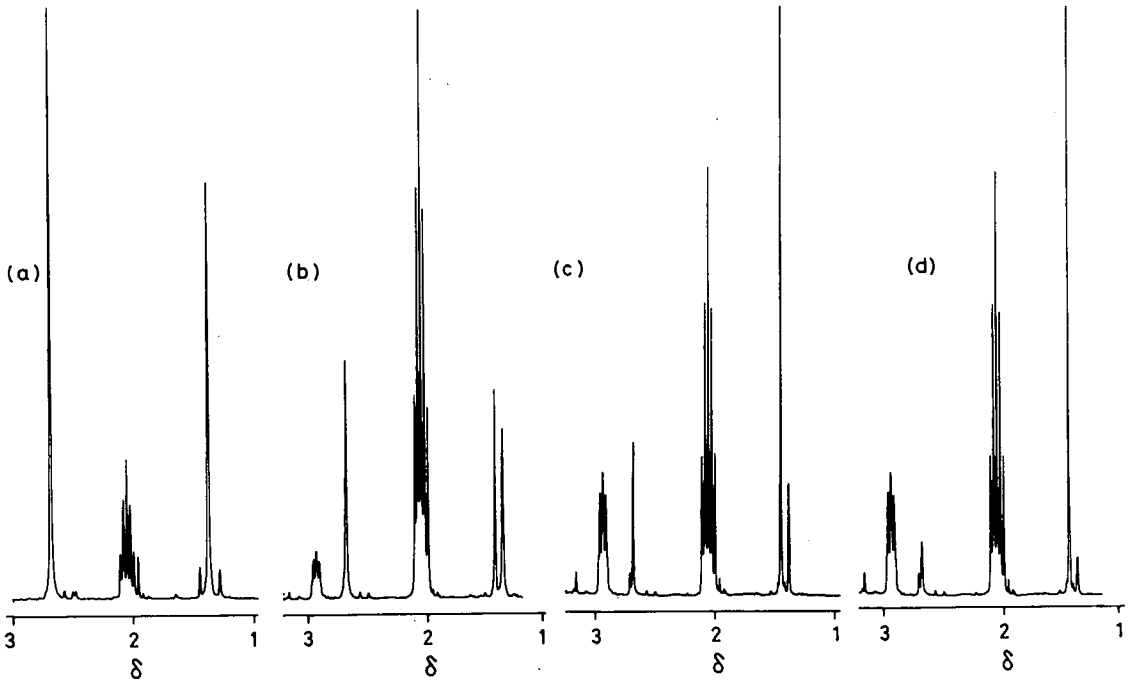

Figure 1. 80 MHz ^{1}H n.m.r. spectra of the reaction mixture from 3-hydroxy-3-methylpentane-1,5-dioic acid (**3**) with acetic anhydride (14 equiv.) at room temperature. Samples were taken at (a) $t = 0$, (b) $t = 2$, (c) $t = 4$, and (d) $t = 6$ h. The excess of acetic anhydride was removed under reduced pressure and the resulting white solid was dissolved in [$^2H_6$]acetone and the spectra determined on a Bruker WP80 spectrometer.

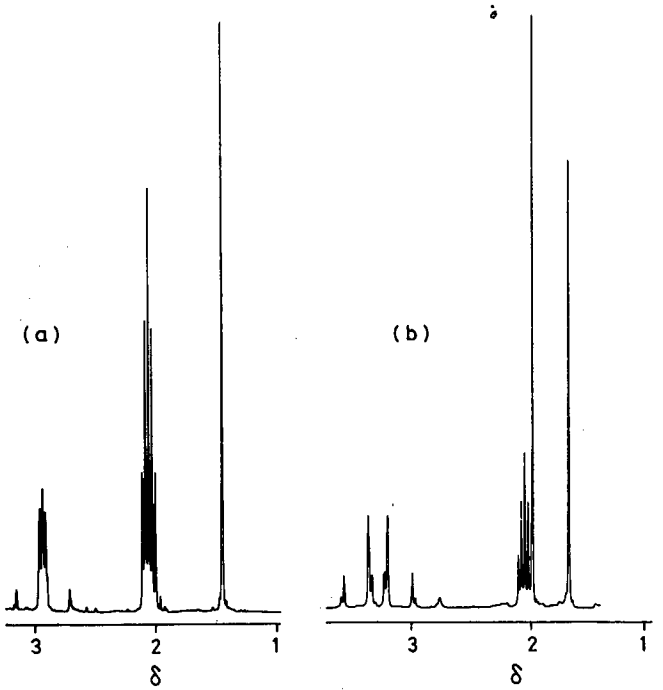

Figure 2. 80 MHz ^{1}H n.m.r. spectra in [$^2H_6$]acetone of (a) 3-hydroxy-3-methylpentane-1,5-dioic anhydride (**4**), and (b) 3-acetoxy-3-methylpentane-1,5-dioic anhydride (**5**).

Experimental

M.p.s. were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H N.m.r. spectra were determined on either Varian EM360, Bruker WP80, or Bruker WP200 spectrometers for deuteriochloroform or hexadeuterioacetone solution. I.r. spectra were determined on a Perkin-Elmer 257 spectrophotometer as KBr discs.

Conversion of Sodium Acetate into n-Butyl Acetate.—Sodium acetate (5 g, 60 mmol) was mixed with tri-n-butyl phosphate (20 ml) and the mixture was heated under reflux for 5 h on an oil-bath at 200—220 °C. The viscous mixture was cooled to room temperature, the upper end of the reflux condenser was sealed through a liquid-nitrogen-cooled trap to a vacuum pump, and the product ester was distilled into the cold trap by heating the reaction flask to 100—160 °C for 2.5 h at 1 mmHg pressure with cold water running in the reflux condenser. n-Butyl acetate (6.8 g, 96%) was obtained.

4-Hydroxy-4-methylhepta-1,6-diene (**2**).—A mixture of ethyl acetate (0.5 g) and allyl bromide (2.06 g) in diethyl ether–THF (1:1; 10 ml) was added dropwise to a stirred mixture of magnesium turnings (0.55 g) in diethyl ether–THF (1:1; 2 ml). After the mixture had been stirred overnight, crushed ice (7 g) was added and the mixture was acidified with 6M-sulphuric acid. The resulting solution was extracted with diethyl ether, and the extract was washed with saturated potassium hydrogen carbonate solution and dried over Na_2SO_4. Removal of the solvent gave a yellow oil which was distilled at water-pump pressure to yield the dienol (**2**) as an oil (0.73 g), b.p. 90—92 °C; $\delta_H(CDCl_3)$ 1.18 (3 H, s), 1.8 (1 H, br s, exchangeable), 2.23 (4 H, d, J 7 Hz), and 5.00—6.00 (6 H, m).

3-Hydroxy-3-methylpentane-1,5-dioic Acid (**3**).—Ozone was passed through a stirred solution of the alcohol (**2**) (0.51 g) in a mixture of methylene dichloride and acetic acid (10:1; 11 ml) at −78 °C until a blue colour appeared. The reaction mixture was then allowed to warm up to room temperature and acetic acid (10 ml) was added. After concentration of the reaction mixture to about 5 ml, more acetic acid (10 ml) and a 30% solution of hydrogen peroxide (4 ml) were added and the mixture was heated under a reflux for 13 h. Evaporation of the solvent gave the diacid (**3**) as an oil (0.55 g) which slowly solidified. Recrystallisation from diethyl ether gave the acid as needles, m.p. 110—111 °C (lit.,[3] 110—111 °C); $\delta_H[(CD_3)_2CO]$ 1.38 (3 H, s),

2.68 (4 H, s), and 6.15 (*ca.* 2 H, br s). Treatment with diazomethane gave the dimethyl ester which showed $\delta_H(CDCl_3)$ 1.39 (3 H, s), 2.73 (4 H, s), and 3.75 (6 H, s).

Reactions of 3-*Hydroxy*-3-*methylpentane*-1,5-*dioic acid* (**3**) *with Acetic Anhydride.*—These were carried out using a wide variety of relative concentrations of diacid (**3**) to acetic anhydride (1:1 to 1:25). Some representative experiments are detailed below.

(a) 14 *Equivalents of acetic anhydride at* 18 °C. A mixture of diacid (**3**) (810 mg, 5 mmol) and acetic anhydride (6.6 ml, 70 mmol) was stirred at room temperature. After 72 h the initial suspension had turned into a clear solution. The excess of acetic anhydride was removed under high vacuum to give a white solid (817 mg) which was recrystallised from diethyl ether–light petroleum (b.p. 30—40 °C) to give 3-hydroxy-3-methylpentane-1,5-dioic anhydride (**4**) as needles (673 mg), m.p. 102—103 °C (lit.,[9] 101—102.5 °C); $\nu_{max.}$ 3 320, 1 810, 1 768, and 1 755 cm^{-1}; δ_H (200 MHz) 1.44 (3 H, s), 2.80, 2.90, 2.98 and 3.06 (4 H, AA'BB'), and 4.67 (1 H, br s, exchangeable) (Found: C, 50.1; H, 5.45. Calc. for $C_6H_8O_4$: C, 50.00; H, 5.56%). Similar results were obtained using between 5 and 20 equivalents of acetic anhydride.

(b) 1.5 *Equivalents of acetic anhydride at* 100 °C. A solution of the diacid (**3**) (405 mg) in acetic anhydride (0.35 ml) was stirred and heated at 100 °C for 1.5 h. After 45 min a clear solution was obtained. Removal of excess of acetic anhydride gave a pale solid which was recrystallised as above to give the anhydride (**4**) as needles (300 mg).

(c) 25 *Equivalents of acetic anhydride at* 18 °C. Reaction between the diacid (**3**) (162 mg, 1 mmol) and acetic anhydride (2.36 ml, 25 mmol) at room temperature gave a clear solution after the mixture had been stirred for 12 h. Removal of excess of acetic anhydride gave a solid (216 mg) which was recrystallised from diethyl ether to give 3-acetoxy-3-methylpentane-1,5-dioic acid anhydride (**5**) as prisms (180 mg), m.p. 83—85 °C (lit.,[10] 85 °C); $\nu_{max.}$ 1 810, 1 775, 1 760, and 1 735 cm^{-1}; δ_H (80 MHz) 1.68 (3 H, s), 1.98 (3, H, s), and 2.99, 3.20, 3.36 and 3.56 (4 H, AA'BB').

(d) 14 *Equivalents of acetic anhydride at* 40 °C. The diacid (**3**) (405 mg, 2.5 mmol) in acetic anhydride (3.3 ml, 35 mmol) was stirred and heated at 40 °C. The usual work-up gave a pale solid (392 mg) which was shown by n.m.r. spectroscopy to consist of a 2:1 mixture of the acetate (**5**) and the anhydride (**4**).

Reaction of 3-*Hydroxy*-3-*methylpentane*-1,5-*dioic Acid* (**3**) *with Acetyl Chloride and Triethylamine.*—Triethylamine (0.35 ml) was added to a solution of the diacid (**3**) (405 mg) in dry THF (30 ml) and the mixture was cooled to 0 °C in an ice-bath. A solution of acetyl chloride (0.2 ml) in dry (20 ml) was then added dropwise to the stirred mixture during *ca.* 5 min. The resulting suspension was stirred for 2 h at 0 °C. The solid residue was filtered off and washed with (2 ml). The filtrate and washings were then concentrated at 30 °C on a rotary evaporator to give a pale pink solid (415 mg) which was recrystallised from diethyl ether–light petroleum (b.p 30–40 °C) to give the anhydride (**4**) as needles (350 mg), m.p. 102—103 °C.

Reaction of 3-*Hydroxy*-3-*methylpentane*-1,5-*dioic Acid* (**3**) *with Acetyl Chloride.*—Diacid (**3**) (0.6 g) was refluxed in acetyl chloride (5 ml) for 4 h. The excess of acetyl chloride was removed under reduced pressure to give a pale solid which was recrystallised from diethyl ether to give the acetate (**5**) as crystals (0.55 g), m.p. 75—76 °C.

Mevalonic Acid Lactone (**1**).—The crude anhydride (**4**) (0.7 g) was dissolved in propan-2-ol (20 ml) and the solution was added dropwise to sodium borohydride (0.4 g) cooled in an ice-bath. The reaction mixture was stirred overnight at room temperature. After removal of the solvent, water (10 ml) was added and the mixture was acidified to pH 2 in an ice-bath. The solution was extracted continuously with diethyl ether for 45 h. The extract was dried (Na_2SO_4) and the solvent removed on a rotary evaporator to give an oil which was shown by t.l.c. to have one component, corresponding to mevalonic acid lactone. Column chromatography on Malinckrodt silica AR-CC-7 (20 g) and elution with hexane–diethyl ether (2:8) gave pure mevalonic acid lactone (**1**) (300 mg).

Acknowledgements

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Biosynthesis of Tajixanthone and Shamixanthone by *Aspergillus variecolor:* Incorporation of Oxygen-18 Gas

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Mass spectral and ^{13}C n.m.r. analyses of tajixanthone (**1**) and shamixanthone (**2**) formed during growth of *Aspergillus variecolor* under atmospheres containing [$^{18}O_2$] oxygen gas showed incorporation of four and three ^{18}O labels per molecule of (**1**) and (**2**), respectively, and provided information about the mode of xanthone ring formation.

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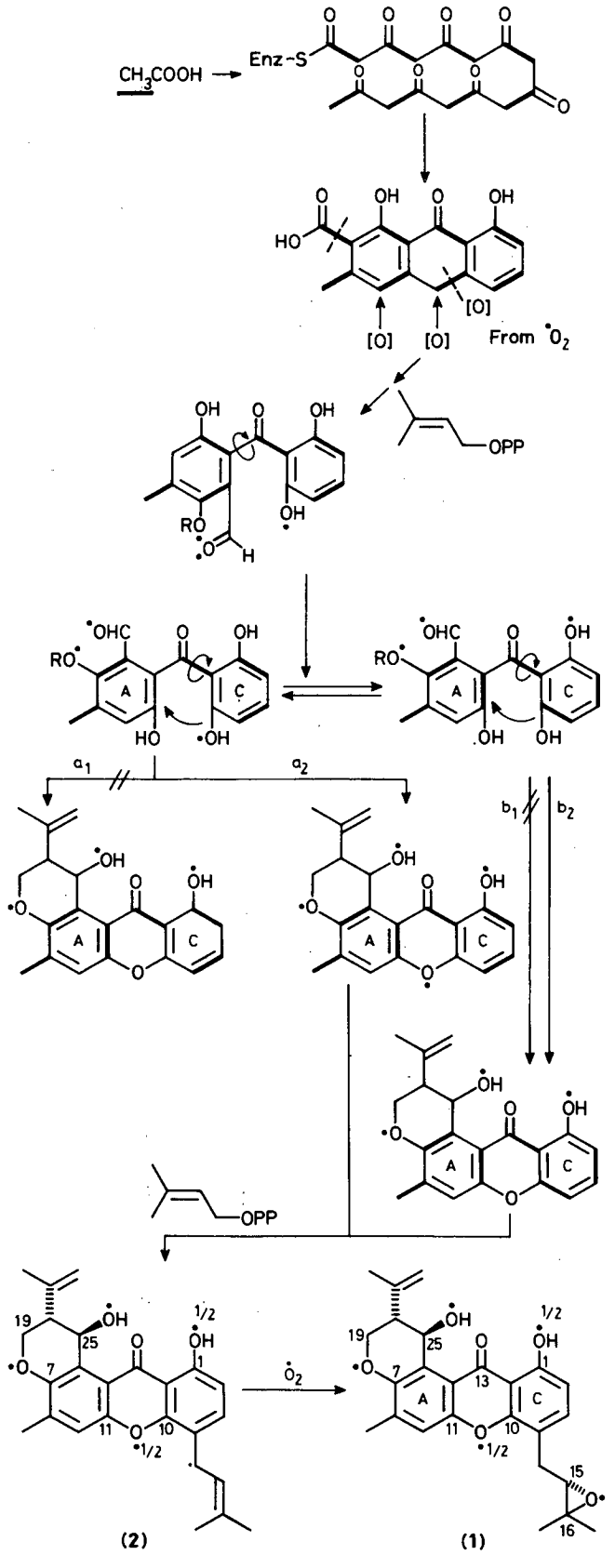
Mycelial pigments like tajixanthone (**1**) and shamixanthone (**2**)[1] as well as various meroterpenoids[2] illustrate how *Aspergillus* species can combine polyketide and terpenoid precursors to form secondary metabolites which have often undergone extensive oxidative elaboration. The isolation of a number of closely related xanthones[3—5] and ^{13}C and ^{2}H labelling studies[6,7] on tajixanthone strongly support the biosynthetic pathway outlined in Scheme 1. Carbon labelling results suggest that an acetate-derived octaketide precursor cyclizes to an anthrone which is hydroxylated, *O*-prenylated by dimethylallyl pyrophosphate, and oxidatively cleaved to a benzophenone derivative, either directly or after oxidation to an anthraquinone. Observation of two distinct carbon labelling patterns present in equal amounts in ring c of (**1**) implies

Table 1. ^{18}O Isotopically-shifted resonances in the ^{13}C n.m.r. spectra[a] of tajixanthone (**1**) and shamixanthone (**2**).

Carbon	δ (**1**)	Δδ (**1**) (× 100)	^{16}O : ^{18}O (**1**)[d]	δ (**2**)	Δδ (**2**) (× 100)	^{16}O : ^{18}O (**2**)[d]
13	184.0	2.7[b]				
1	160.4[c]	1.0	79 : 21	159.7	1.0[e]	
10	152.9[c]	2.3	80 : 20	152.8	2.5	71 : 29
11	152.0	2.4	77 : 23	152.2	2.3	73 : 27
7	149.5	1.5	61 : 39	149.4	1.6	53 : 47
19	64.5	2.3	66 : 34	64.5	2.5	55 : 45
15	63.24	3.3	64 : 36			
25	63.16	1.5	66 : 34	63.2	1.5	60 : 40
16	58.5	4.1	64 : 36			

[a] Spectra run at 100.6 and 90.6 MHz; for experimental conditions see ref. 9. [b] Enriched by sodium [1-^{13}C,$^{18}O_2$]acetate only; all others enriched by $^{18}O_2$. [c] These assignments were originally reversed in ref. 6. [d] Approximate (±5%) ratios from peak areas. [e] Not resolved completely.



R = $CH_2CH{=}CMe_2$
OPP = Pyrophosphate
Scheme 1

the intermediacy of a symmetrical dihydroxyphenyl moiety which is free to rotate prior to cyclization to a xanthone.[7] Since the detection of ^{18}O-induced isotope shifts in ^{13}C n.m.r.[8] has proved useful in determining the mode of xanthone ring formation in ravenelin[9] and sterigmatocystin,[10] we have studied the incorporation of $^{18}O_2$ gas into tajixanthone (**1**) and shamixanthone (**2**).

A fermentation of *Aspergillus variecolor*[1] in which the normal atmosphere was replaced with one containing $^{18}O_2$ gas (98.7% isotopic purity) gave tajixanthone (**1**), the mass spectrum of which showed the presence of four ^{18}O atoms per molecule. The 100.6 and 90.6 MHz ^{13}C n.m.r. spectra of a mixture of this and unlabelled material displayed isotopically-shifted resonances for eight of the nine oxygen-bearing carbons (Table 1). Only the carbonyl oxygen at C-13 remained completely unlabelled in this experiment. Within experimental error, the relative amount of ^{18}O incorporated at C-1 and at C-10 is half of that at the other labelled sites. Taken together with the mass spectral results, this shows that in a particular molecule of tajixanthone (**1**) either the oxygen at C-1 or the one at C-10 was labelled, but not both. This confirms the intermediacy and oxidative origin of a conformationally labile benzophenone which has an axis of symmetry in a dihydroxyphenyl ring. More importantly, the results demonstrate that xanthone ring closure must proceed almost exclusively by a Michael addition–elimination[11] process in which the ring C oxygen attacks the ring A carbon with ultimate loss of the ring A oxygen at C-11 (paths a_2 and b_2). Cyclization in the opposite sense with retention of the ring A oxygen (paths a_1 and b_1) is very minor if it occurs at all.

The presence of ^{18}O at C-25 and the previously reported loss of ^2H from acetate at that position[7] suggest oxidative cleavage of an anthraquinone rather than anthrone precursor. Mass spectral analysis of the molecular ion region of (**1**) obtained from a fermentation utilizing a mixture of $^{16}O_2$ and $^{18}O_2$ shows that each aerobically-derived oxygen atom is introduced separately by mono-oxygenation. Thus the involvement of dioxygenase-derived dioxetanes[12] or endoperoxides[13,14] which have been proposed as intermediates in the cleavage mechanism can be ruled out. Presumably cleavage occurs *via* a biological Baeyer-Villiger type oxidation[15] to give an intermediate lactone which can undergo direct reduction to the hemiacetal (*cf.* arugosin A/B[3]) and thence to the benzophenone.

In a separate experiment sodium [1-^{13}C,$^{18}O_2$]acetate (90% ^{18}O) was fed to cultures of *A. variecolor* grown in a normal atmosphere, and the resulting tajixanthone (**1**) was analysed by ^{13}C n.m.r. Unfortunately the incorporation level was too low to detect isotope shifts at any carbons except C-13, the C–O bond of which was thereby shown to be acetate-derived.

As expected, shamixanthone (**2**) isolated in the same experiment with $^{18}O_2$ showed, by mass spectral analysis, the incorporation of three ^{18}O atoms per molecule. Although the isotope shift in the ^{13}C n.m.r. of (**2**) at C-1 could not be completely resolved for accurate determination of the ^{16}O : ^{18}O ratio, the presence of ^{18}O at that site and the reduced ^{18}O content of the xanthone ring oxygen relative to other sites (Table 1) confirm the operation of the same biosynthetic pathway as that of tajixanthone (**1**). It is interesting to note that in ravenelin biosynthesis the same type of xanthone ring closure (paths a_2 and b_2) occurs with retention of the oxygens of a symmetrical dihydroxyphenyl moiety.[9] In contrast, retention of oxygen from the other ring and a single carbon labelling pattern during sterigmatocystin biosynthesis[10] suggest an oxidative coupling mechanism rather than addition–elimination for xanthone formation in that case.[16,17]

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